BIOINSECTICIDES FROM INSECT NATURAL ANTAGONISTS

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Dottorato in Biotecnologie – XXXV ciclo

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SUMMARY

Ecological and economic sustainability of agricultural production is significantly dependent on the availability of alternative plant protection tools and strategies to replace synthetic pesticides. Bioprotection of crop plants, which includes the use of both biocontrol agents and non-living plant protection tools originated from nature, is the most valid option currently being pursued by the scientific community. Of particular interest is the exploitation of pest suppression mechanisms that insect natural antagonists have evolved, to develop “bio-inspired” pest control measures. This PhD thesis aims to contribute to this research area, by developing new bioinsecticide molecules from virulence factors that natural enemies of pest insects use to subdue their victims.

The first part of the thesis focuses on a novel reverse genetic approach *in vivo* for functional analysis of a venom protein in the aphid parasitoid, *Aphidius ervi*. RNAi-mediated silencing of the gene encoding the most abundant component of the venom, *Ae*-γ-glutamyl transpeptidase (*Ae*-γ-GT), was used to score its impact on parasitized host and parasitoid’s progeny. Our results revealed a new function for this key virulence factor, which plays a crucial role in the fine regulation of the complex interactions between the wasp’s progeny, the host, and its primary bacterial symbiont, *Buchnera aphidicola*, with important consequences on host nutritional suitability for the developing parasitoid larvae.

In the second part, the insecticidal activity of *Tn*BVANK1, a virulence factor belonging to the ANK protein family of the Polydnavirus associated with the parasitoid *Toxoneuron nigriceps*, was evaluated against aphids, as a basis to develop new pest control strategies for plant sap-sucking insects. This is very much needed, since very few natural molecules are currently available for controlling these pests. The virulence factor considered was orally toxic for the aphid species *Acyrthosiphon pisum*, exerting a negative impact on the structure and integrity of midgut epithelium.

Finally, in the third part, a fusion protein containing a neurotoxic peptide from the venom of a spider and the bovine serum albumin (BSA), as a carrier moiety for mediating transepithelial transport across the midgut barrier, was tested for its oral toxicity against economically important pests in the order Lepidoptera, Hemiptera and Diptera. The obtained results validated the fusion strategy adopted for the delivery of this selective and effective spider toxin.

The overall results of this PhD study contribute to the development of new and effective bioprotection tools, providing nice examples on learning from Nature how to control pest insects.

RIASSUNTO

Il controllo degli insetti dannosi in agricoltura è essenziale per garantire la produzione di cibo sicuro e in quantità adeguate a sostenere la crescente popolazione mondiale. Tuttavia, l'uso eccessivo di insetticidi chimici di sintesi ha forti ripercussioni sull'ambiente e gli organismi non bersaglio. Per questo motivo, è fondamentale ridurre l'uso di queste sostanze e privilegiare l'adozione di strategie di controllo integrato, basate sull'uso di antagonisti naturali e di prodotti alternativi a quelli convenzionali. Questi ultimi sono di vario tipo, in maggioranza è di origine naturale, spesso contenenti antagonisti naturali o molecole da essi prodotte, caratterizzate da elevata selettività e ridotta persistenza ambientale, in modo da consentire un basso impatto sugli organismi non bersaglio.

Il crescente interesse in quest'area di ricerca ha permesso di ampliare in modo significativo le conoscenze sulle tossine ad attività insetticida prodotte da antagonisti naturali di insetti, che oggi includono una serie di nuove ed efficaci molecole, impiegabili come alternative alla già consolidata tossina prodotta dal batterio entomopatogeno *Bacillus thuringiensis* (Bt). Tra le fonti di nuove molecole ad attività insetticida spiccano gli entomofagi dotati di veleno, come i ragni che iniettano potenti neurotossine per paralizzare le loro prede, o i parassitoidi, che utilizzano diversi fattori di virulenza per uccidere o manipolare la fisiologia e lo sviluppo delle loro vittime. Pertanto, questo gruppo di artropodi fornisce l'opportunità di identificare una serie di molecole potenzialmente utilizzabili per lo sviluppo di nuovi biopesticidi, la cui fruizione è ampiamente dipendente dalla messa a punto di adeguate strategie di rilascio ambientale, finalizzate a contenere la loro degradazione e a favorirne l'assorbimento da parte degli insetti bersaglio.

Nella prima parte del dottorato abbiamo concentrato le nostre attività di ricerca sull'associazione tra l'afide delle leguminose, *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae), e il parassitoide endofago *Aphidius ervi* Haliday (Hymenoptera: Braconidae), un sistema modello largamente utilizzato per studiare i meccanismi molecolari alla base delle interazioni complesse tra parassitoidi e loro ospiti. Utilizzando la tecnica dell'RNA interferente (RNAi), è stato studiato *in vivo* il ruolo funzionale della componente proteica più abbondante del veleno di *A. ervi*, *Ae*-γ-glutamyl transpeptidasi (*Ae*-γ-GT), un enzima coinvolto nel metabolismo del glutatione la cui alterazione determina una risposta apoptotica nei tessuti ovarici (germari) e la conseguente castrazione dell'ospite. Le microiniezioni di RNA a doppio filamento (dsRNA) nelle

pupe di femmine di *A. ervi* hanno permesso di silenziare stabilmente l'espressione di *Ae*-γ-GT nello stadio adulto. Al silenziamento sono risultati associati cambiamenti fenotipici sia negli afidi parassitizzati, sia nella progenie del parassitoide. Misurazioni morfometriche hanno rilevato un inaspettato incremento delle dimensioni sia dell'ospite che del parassitoide, associato a un aumento della carica batterica del simbionte primario dell'afide, *Buchnera aphidicola*. La progenie adulta, derivante da femmine di *A. ervi* silenziate, ha mostrato una ridotta longevità in entrambi i sessi, mentre le femmine hanno riportato tassi riproduttivi inferiori rispetto al gruppo di controllo. Le analisi effettuate sui tessuti riproduttivi dell'afide hanno evidenziato il ruolo primario di *Ae*-γ-GT nella degenerazione ovarica dell'ospite, indicando che questa proteina controbilancia la proliferazione di *Buchnera*, probabilmente innescata da altri componenti del veleno del parassitoide. Il nostro studio, inoltre, fornisce un nuovo approccio per studiare la complessità del veleno dei parassitoidi degli afidi *in vivo* e fa luce su un nuovo ruolo di *Ae*-γ-GT nella regolazione dell'ospite.
In futuro, ulteriori caratterizzazioni funzionali, sia *in vivo* che *in vitro*, utilizzando la proteina *Ae*-γ-GT ricombinante, consentiranno di ampliare le nostre conoscenze sui meccanismi molecolari alla base degli effetti osservati, un ulteriore passo verso lo sviluppo di questa componente del veleno come potenziale molecola bioinsetticida.
La seconda e la terza parte della tesi riguardano studi condotti su molecole derivanti da artropodi antagonisti di insetti, come parassitoidi e ragni, per valutare la loro potenziale attività insetticida. Abbiamo utilizzato la tecnologia delle proteine ricombinanti per produrre queste molecole e le abbiamo saggiate sia da sole che in combinazione con vettori specifici, come la BSA, valutando l'assorbimento e la tossicità orale *in vivo*
Il fattore di virulenza del Polidnavirus associato al parassitoide *Toxoneuron nigriceps*, chiamato *TnBVANK1*, è coinvolto sia nell'immunosoppressione, sia nell'interruzione della biosintesi dell'ecdisone nell'ospite parassitizzato.
Considerando che studi preliminari su *Tn*BVANK1 hanno riportato tossicità orale della proteina nei confronti del lepidottero nottuide *S. littoralis,* nel presente lavoro abbiamo saggiato l'effetto tossico orale di *Tn*BVANK1 sull'afide delle leguminose *A. pisum,* analizzando gli effetti della proteina sull'intestino degli insetti, attraverso osservazioni di microscopia ottica ed elettronica a trasmissione (TEM), allo scopo di valutarne i possibili effetti su insetti succhiatori di linfa, per i quali non abbiamo efficaci molecole naturali adottabili per lo sviluppo d nuove tecnologie di controllo.

I risultati hanno mostrato che *Tn*BVANK1 è significativamente tossico per *A. pisum* quando somministrato per via orale e che l'ingestione della proteina causa danni alla struttura della regione prossimale dell'intestino tubolare degli insetti.
Questi risultati supportano l'utilizzo di *Tn*BVANK1 come candidato per lo sviluppo di nuove strategie di controllo degli insetti succhiatori di linfa, come gli afidi. In futuro, saranno necessarie ulteriori indagini per sviluppare adeguati sistemi di rilascio in campo e per valutare adeguatamente il rischio associato al suo uso.
La terza parte della tesi descrive lo studio effettuato su una neurotossina prodotta dalle ghiandole salivari del ragno *Segestria florentina*, chiamata SFl6, fusa con l'albumina del siero bovino (BSA). La neurotossina SFl6 è nota per essere un antagonista altamente selettivo di diversi canali del calcio nel sistema nervoso centrale degli insetti, mentre la BSA è stata utilizzata per consentire il trasporto transepiteliale della tossina sttraverso la barriera gastrica della tossina, avendo dimostrato di attraversare efficacemente l'epitelio intestinale delle larve di lepidotteri in forma non degradata. È stata saggiata, quindi, l'attività biologica della proteina di fusione ricombinante SFl6-BSA, costituita dalla SFl6 legata all'estremità N-terminale della BSA, su diverse specie di insetti. La proteina di fusione è risultata significativamente tossica in seguito a somministrazione orale nei confronti di fitofagi appartenenti a diversi ordini, come *S. littoralis*, *A. pisum* e *Ceratitis capitata*, suggerendo che un approccio basato sulle proteine di fusione può offrire l'opportunità di migliorare significativamente l'efficacia orale di tossine attive a livello emocelico. Si può, quindi, concludere che la proteina di fusione SFl6-BSA rappresenta una valida strategia di controllo, attiva nei confronti di diverse specie di notevole rilevanza economica.

1 BACKGROUND

1.1 Integrated Pest Management (IPM) and biological control strategies

Interest in productive and environmentally sustainable agriculture has increased over the years and fostered the development of technologies aimed at increasing productivity and reducing the negative impact of farming practices (Flint and Van den Bosch, 2012; Deguine et al., 2021). Long-term use of chemicals (including insecticides, fungicides, and herbicides) has generated genetic resistance in pests, resulting in increased application rates, higher crop losses, and higher costs to farmers (Oerke, 2006; Pimentel and Burgess, 2014; Mahmood et al., 2016). Massive use of pesticides is also associated with high health risks for the exposed population, mainly farmers and agricultural workers, as well as for the rural population and consumers (Bergman, 2013; Kim et al., 2017). The use of agrochemicals causes pollution, with negative impacts on soil system health, water quality, and natural reserves (Wightwick et al., 2010; Stehle and Schulz, 2015). With the advent of the Green Deal and in particular the Farm to Fork and Biodiversity strategies (European Commission, 2019), one of the main challenges facing agriculture is to achieve safe food production and reduce the use and risk of pesticides by 50% by 2030. Achieving this goal, however, is difficult without developing viable alternatives.

Over the past six decades, Integrated Pest Management (IPM) has evolved into an environmentally sound crop protection strategy (Deguine et al., 2021). In an IPM approach, careful consideration of all available pest control techniques and subsequent integration of appropriate measures that inhibit the development of harmful organism populations prevails (Peterson et al., 2018; FAO, 2020). In this way, chemical pesticides remain at economically acceptable levels and minimize risks to human health and the environment, promoting the growth of a healthy crop with minimal disruption of agricultural ecosystems and supporting natural pest control mechanisms (Dara, 2019; FAO, 2020).

Biological control is an important part of IPM programs and is a form of ecologically based pest control that uses one type of organism to control another (Hajek and Eilenberg, 2018). Natural enemies of insect pests include parasitoids, predators, entomopathogenic nematodes and pathogens (Baker et al., 2020). Biological approaches also include pheromones, sterile insect release, and biopesticides,

which are formulations of pesticides based on, or derived from, living organisms (Dhakal and Singh, 2019; Baker et al., 2020). The International Biocontrol Manufacturers' Association (IBMA) promotes the broader term *bioprotection,* which includes the use of both biocontrol agents and non-living plant protection tools originated from nature (Stenberg et al., 2021). The growing knowledge on the functional basis of biological control allows to include in this definition also the use of molecules/genes deriving from natural antagonists, which are able to reproduce the lethal syndrome they induce in the target pests they use as hosts (Bale et al., 2008; Pennacchio et al., 2012; Mahmood et al., 2016). A large number of bioactive molecules, that regulate insect antagonistic interactions, have been already isolated and characterized from plants and microorganisms (bacteria, fungi, and viruses), to obtain insecticides of natural origins (Kachhawa, 2017), and many untapped sources are available in nature.

Parasitic wasps (Insecta, Hymenoptera) are the most effective natural enemies of insects. The astonishing diversity of developmental patterns and host regulation strategies displayed by these parasitic Hymenoptera provides the opportunity to identify new molecules and genes involved in a number of pathologies induced in the host (Beck et al, 2000; Pennacchio and Strand, 2006), which are ideal candidates for the development of new bioinsecticides (Bravo et al., 2007; Pennacchio et al., 2012). This novel approach expands the concept of biological control, since it is based on the use of natural antagonists beyond the organism level, as a source of bioinspired tools and strategies for crop protection.

1.2 Biotechnology for plant protection

Biotechnological advance in agriculture stems from earlier progresses in genetic engineering and molecular biology that have led to the identification, isolation, characterization, and modification of genes from different biological sources (Kumary et al., 2022). This has enabled the production of a variety of biotechnology-based solutions for pest control, which include the use of biopesticides (Pérez-García et al., 2011; Kumary et al., 2022). Biopesticides are crop protection products that are developed by naturally occurring living organisms such as animals, plants, and microorganisms (e.g., bacteria, fungi, and viruses) (Dhakal and Singh, 2019; Baker et al., 2020). Biopesticides can control serious harmful organisms to agriculture

and, due to their ecological and specific mode of action, are gaining in importance worldwide (Baker et al., 2020).
One of the first commercialized biopesticides were based on the use of the entomopathogenic bacteria *Bacillus thuringiensis* and its derived toxins, both for direct application and for developing transgenic crops (Roh et al., 2007). Similarly, toxins like spinosyns (Sparks et al., 2001) are other examples of natural insecticides of wide use in IPM derived from bacteria, while a number of natural compounds, of plant origin (Miresmailli et al., 2014) or produced by predatory arthropods (Smith et al., 2013), are increasingly used or look particularly promising for developing new control tools. In the last years, RNA interference (RNAi) mediated by double-stranded RNA (dsRNA) delivery has emerged as a powerful technique for down-regulating gene expression in insects (Xue et al., 2012). Beside its importance in reverse genetics studies, RNAi has a great potential for pest control, with different options for its application in the field, including transgenic plants expressing the insecticidal RNAi trait (plant-mediated RNAi) and novel dsRNA-nanoconjugate formulations to be applied as a traditional insecticide (Xue et al., 2012; Lundgren and Duan 2013). RNAi offers exquisite specificity and flexibility that cannot be matched by other crop protection interventions such as chemical insecticides, biological control, or protein-coding transgenes (Scott et al. 2013).

1.3 Parasitoid wasps and host regulation

Parasitoids are largely represented by the order of the Hymenoptera (parasitic wasps) (Quicke, 1997), in which the largest and most conspicuous species generally belong to the Ichneumonoidea, a superfamily that includes the Ichneumonidae and the Braconidae (Gómez et al., 2018). Adult wasps are free-living, while juveniles have a parasitic lifestyle, feeding on different life stages of their host insects (Brodeur and Boivin, 2004; Wajnberg and Colazza, 2013). The female parasitoid lays the eggs on (ectoparasitoid) or inside (endoparasitoid) the body of another insect (the host); then, the hatching larvae complete their development by feeding on the host tissues (Godfray, 1994). This feeding activity kills the host, and, finally, the wasp's larva develops into a pupa, from which an adult emerges (Wajnberg and Colazza, 2013).
Parasitic wasps display a wide range of host exploitation strategies to regulate and redirect the food source to their own advantage (Godfray, 1994; Pennacchio and Strand, 2006).

Idiobiosis and koinobiosis are two kinds of host utilization strategiess that are usually used to categorize parasitoids (Harvey, 2005; Mackauer and Sequeira 1993). Idiobiont parasitoids paralyze their host or develop on immobile stages such as eggs and pupae (Pennacchio and Strand, 2006). Koinobiont wasps, on the other hand, evolve into a growing host that continues to move and feed for a significant period of the parasitoid larval development (Pennacchio and Strand, 2006; Cuny and Poelman 2022). Koinobionts are nearly in all cases endoparasitoids and are thought to be more specialized than idiobionts, which usually are ectoparasitoids (Santos and Quicke, 2011; Cuny and Poelman 2022).

Life history strategies among parasitic wasps are extremely diverse and can be quite intricate, often with finely tuned associations between parasitoids and hosts (Henri and Van Veen, 2011). Parasitism induces changes in host nutritional physiology and appears to be critical to wasp fitness (Pennacchio and Strand, 2006). Indeed, parasitoids show surprising adaptive plasticity with respect to host size constrains, nutrient uptake limitations, and variations in host availability (Jervis, 2008). Favorable larval feeding conditions lead to an increase in offspring size and its related traits such as fecundity and longevity (Mackauer and Sequeira, 1993; Ellers and Jervis, 2003). Changes in parasite fitness attributes in response to variations in host resources are indicative of the parasite life-history strategy (Mackauer and Sequeira, 1993; Jervis, 2008). In several koinobiont species, parasitism induces biochemical changes in the host's hemolymph (Harvey and Malcicka, 2016), thereby increasing the bioavailability of nutrients as proteins, lipids, and glucose, to support the development of the wasp larva (Jervis, 2008; Harvey and Malcicka, 2016). These changes are the result of the host regulation exerted by parasitoid wasps to enhance the development their offspring (Vinson and Iwantsch, 1980). The finest examples of such regulation strategies occur in some koinobiont species whose larvae are able to synchronize their molt with the molt of their hosts (Beckage and Gelman, 2004).

Host regulation also includes multiple impacts on host immunity, which is the first physiological barrier encountered by endophagous parasitoids after invading host's hemocoel (Pennacchio and Strand, 2006). To regulate host immunity and physiology, parasitoids produce and release regulation factors, of maternal or embryonic origin, in the host hemocoel (Pennacchio et al., 2014; Beckage and Gelman, 2004). Maternal regulation factors are released by female wasps during oviposition (Poirié et al., 2009; Pennacchio et al., 2014) and include

venom (Asgari and Schmidt, 1994; Webb and Luckhart, 1994,1996; Tanaka et al., 2002), ovarian proteins (Asgari and Schmidt, 1994) and polydnaviruses (PDVs) (Webb et al., 2000; Webb and Strand, 2005), while embryonic factors include teratocytes (a specific cell type derived from the embryonic serosal membrane) and parasitoid larva itself (Krell et al., 1982; Dahlman and Vinson, 1993; Jones and Coudron, 1993).

1.3.1 Polydnaviruses

Many parasitoids harbour endogenous viruses from the family of the Polydnaviridae that replicate only in the calyx of adult wasps and are injected inside the host during oviposition (Pennacchio and Strand, 2006; Strand and Burke, 2019). The association with viruses arose in two separate lineages of parasitoids belonging to the Braconidae and Ichneumonidae families (Strand and Burke 2015). The polydnaviruses (PDVs) are therefore divided into two groups: bracoviruses and ichnoviruses (Strand and Burke 2015; Cuny and Poelman, 2022). Once released into the host, PDVs infect the host cells and discharge their DNA into the nuclei where the expression of viral gene products has several effects on host immunity and physiology, (Pennacchio and Strand, 2006; Yu et al., 2016; Strand and Burke, 2019).

1.3.2 Venom

While parasitizing their host, koinobiont parasitoids also inject non-paralyzing venom, a complex mixture mainly composed of enzymes with diverse functions (Pennacchio and Mancini, 2012; Poirié et al. 2014), which is produced in the parasitoid venom gland and stored in the venom reservoir. Venom injected by koinobiont endoparasitoids typically plays an important immunosuppressive role (Asgari and Rivers 2011; Moreau and Asgari 2015), but also affects the host development (Pennacchio and Mancini, 2012; Pennacchio et al., 2014). For koinobiont parasitoids that harbour PDVs, venom also plays a synergistic role in the support of the PDVs functions (Cuny and Poelman, 2022).

1.3.3 Teratocytes

Some parasitoid species from two families (Braconidae and Platygastroidea) have a specialized embryonic membrane that dissociates at the hatching, giving rise to cells, called "teratocytes", released into the host haemolymph (Dahlman, 1990; Pedata et al., 2003; Strand, 2014). Teratocytes play an important role in the

arrestment of the host growth by producing proteins and miRNAs that interfere with host hormones that control its growth and metamorphosis (Falabella et al., 2000; Wang et al. 2018). Furthermore, in the model parasitoid *Aphidius ervi*, teratocytes have been found to play an important role in the host regulation process (Pennacchio and Mancini, 2012). They release abundant levels of parasitoid-specific proteins into the host hemocoel that provide nutritional support to the developing parasitoid larva and concur in the host castration process (Falabella et al., 2000; Pennacchio and Strand, 2006; Pennacchio et al., 2012).

1.4 RNA interference for studying insect venom functions

RNA interference (RNAi) is a mechanism of transcriptional, post-transcriptional and translational regulation of gene-expression, which is highly conserved among higher eukaryotes (Carthew and Sontheimer, 2009; Berezikov, 2011). A messenger RNA (mRNA), in the presence of complementary RNA (endogenous or exogenous), forms a very stable double-stranded structure, leading to specific degradation of mature mRNA and, therefore, to the block of gene expression (Bartel, 2009).

Shortly following its discovery in the nematode, *Caenorhabditis elegans* (Fire et al., 1998), RNAi has been observed in a wide range of eukaryotic organisms and has proved itself to be a powerful tool for investigating gene function (Dykxhoorn and Lieberman, 2005).

The advent of RNAi also revolutionized the entomological research, as novel gene functions were efficiently discovered (Thankur et al., 2016). In 1998, Kennerdell and Carthew were the first to use RNAi *in vivo* to study the genes *Frizzled* and *Frizzled-2* in *Drosophila melanogaster*. The tremendous success of RNAi in model organisms has prompted its use for research in other insect species as well. In genomics and post-genomics era with the availability of a large amount of sequence information, RNAi further provides an opportunity to investigate the vital functions and crucial interactions that are of importance in agriculture. The research application of RNAi in entomology has elucidated the functions of several genes. Various insect orders have demonstrated amenability to RNAi-mediated silencing. Species belonging to Coleoptera, Lepidoptera, Diptera, Hemiptera, Orthoptera, Blattodea and Hymenoptera orders have been studied for various aspects using RNAi technique (Alamalakala et al., 2018). A large majority of the target genes were gut-specific genes; however, genes from salivary glands, brain and antennae have also

been targeted (Christiaens et al., 2020). RNAi-based studies can be carried out by either in vivo or in vitro studies.
Application of RNAi could also be useful in studying the function of specific venom proteins in host-parasitoid interaction (Moreau and Asagri, 2015). In a recent study, Colinet et al. (2014) utilized RNAi to knockdown *RhoGap* gene, which is abundantly expressed in *Leptopilina boulardi* venom glands. Silencing was achieved by microinjection of dsRNA specific to the gene into the parasitoid pupae. The results showed near complete silencing of the gene and lack of the protein detection in the venom reservoir of the wasps emerged from gene-specific dsRNA injected pupae. Interestingly, the silencing effect remained stable throughout the entire wasps' lifetime. Similarly, Wang et al., (2020) studied the functions an *α*-amylase highly expressed in venom apparatus of the endoparasitic wasp *Pteromalus puparum* using RNAi, demonstrating its influence in the host metabolism that support the development of parasitoid offspring. These results provided a new experimental tool to study *in vivo* the specific role of venom proteins in host-parasitoid interactions and their importance in the success of parasitism.

1.5 Novel biopesticides from insect antagonists

Predators and parasitoids are among the most abundant natural enemies of insects, and commonly used as biocontrol agents (Bale et al., 2008; Wang et al., 2019; Pijnakker et al., 2020). However, recent research has also shown their potential as a source of molecules for the development of new biotechnologies for insect pest control (Pennacchio et al., 2012). These molecules, identified in the venom of parasitoids (Pennacchio and Strand, 2006) and predators with a high level of trophic specialization, may be ideal candidates for the development of highly selective biological insecticides.
Insect parasitoids exhibit high biodiversity due to their ability to adapt to different ecological niches (Pennacchio and Strand, 2006). The development of various virulence strategies in different phylogenetic lineages is a result of this adaptation and include the use of a wide range of molecules with a potential insecticidal activity. These molecules can range from aggressive neurotoxins, produced by idiobiont parasitoids, to host regulation factors, produced by koinobiont species, which disrupt essential physiological functions such as immunity, neuroendocrine balance, and reproduction (Pennacchio and Strand, 2006; Pennacchio et al., 2012).

The molecules responsible for the paralysis of prey, especially those identified in the venoms of spiders and scorpions, have been studied particularly thoroughly because of their medical importance (Zlotkin, 2005; Maggio et al., 2005; Gordon et al., 2007; Gurevitz et al., 2007; Nicholson, 2007; Rohou et al., 2007). These studies have allowed the identification and molecular characterization of highly selective neurotoxins, which target various voltage-gated ion channels in arthropods. The research on these neurotoxins has received increasing attention from the agrochemical industry, interested in developing new insecticides derived from natural molecules (Gurevitz et al., 2007). Interestingly, a recent study on the effects of neuroactive protein derived from spider venom on pollinators did not show negative impacts on *Apis mellifera* populations (Nakasu et al., 2014), indicating the occurrence of a further level of selectivity, very relevant from an ecological point of view.
Molecules produced by spider and parasitoids have been found to have significant potential for the development of new “bio-inspired” technologies for insect pest control. While the identification and molecular characterization of these molecules has been thoroughly investigated, the challenge lies in developing delivery strategies to effectively target the hemocoelic receptors in the host body cavity following oral administration. These delivery strategies will be crucial for the profitable use of the molecular biodiversity of insect natural enemies in the development of selective and highly effective biopesticides.

1.6 Delivery of bioinsecticide molecules

The use of alternative sources of bioactive molecules, including natural insect antagonists, as a means of controlling pest populations has attracted significant attention due to the potential to tap into a largely unexploited source of molecular diversity (Pennacchio et al., 2012). However, the stability of these molecules in the insect gut environment and their absorption in an active form after ingestion represent significant challenges that need to be addressed (Fitches et al., 2001; Jeffers and Roe, 2008). Indeed, the intestinal epithelium acts as a barrier between these insecticidal molecules and their receptors which are usually located in the hemocoel, where they are normally injected by predators and parasitoids. Moreover, the insect gut, characterized by extreme pH conditions and a metabolically active microbiota, represents a hostile environment for bioactive molecules, such as enzymes. Thus, research efforts in this area are

focused on the identification of stabilizers, able to decrease or avoid degradation in the insect gut lumen, and carriers, able to facilitate the entrance of bioinsecticidal molecules into the cells and their transepithelial transport (Pennacchio et al., 2012). As far as the stabilizers, excellent results have been obtained through the PEGylation (covalent bond between the polyethylenglycol and the bioinsecticide molecule). In fact, this technology has been proved to be capable of protecting proteinaceous insecticidal molecules from degradation, increasing their oral toxicity (Jeffers et al., 2012; Jeffers et al., 2014). Regarding carrier molecules, one of the most significant examples is represented by GNA (*Galanthus nivalis* agglutinin). GNA is able to cross unaltered the intestinal barrier of different insects and acts as vector for proteinaceous molecules with hemocoelic targets, such as neuropeptides and neurotoxins produced by spiders and scorpions (Fitches et al., 2002; Fitches et al., 2004).

Another promising carrier is the bovine serum albumin (BSA), which is transported through lepidopteran midgut by transcytosis, as observed in a controlled *in vitro* experimental setting (Casartelli et al., 2005). A subsequent study clarified that the mechanism responsible for BSA internalization in *B. mori* intestinal cells is clathrin-mediated endocytosis, which involves the recognition of BSA by a megalin-like receptor (Casartelli et al., 2008).

The oral intake of macromolecules with insecticide activity remains the main route of entry in most cases (Pennacchio et al., 2012). Thus, the successful delivery of novel bioinsecticide macromolecules requires the use of carriers that facilitate their crossing of intestinal barriers.

OBJECTIVES

The overall objective of this PhD project is to identify and characterize virulence factors used by predators and parasitoids to subdue or kill their victims, with the ultimate goal of developing novel bioinsecticide molecules from these insect antagonists.
This general objective is pursued with the following research activities:

1. Functional characterization of a venom protein from the parasitoid *Aphidius ervi*, to understand its role in host regulation.
2. Evaluation of the oral toxicity on aphids of a virulence factor encoded by a symbiotic polydnavirus associated with a braconid wasp.
3. Development of a fusion protein made of a spider venom neurotoxin and a carrier protein promoting translocation across the gut and assessment of its oral toxicity against agricultural pests of economic importance.

References

Alamalakala, L., Parimi, S., Patel, N., & Char, B. (2018). Insect RNAi: integrating a new tool in the crop protection toolkit. *Trends in insect molecular biology and biotechnology*, 193-232.

Alemu, M. (2020). Applications of biotechnology for characterization of plants and pests as the key components of plant protection and production strategies: A review. *International Journal of Applied Sciences and Biotechnology*, *8*(3), 247-288.Baker, B. P., Green, T. A., & Loker, A. J. (2020). Biological control and integrated pest management in organic and conventional systems. *Biological Control*, *140*, 104095.

Asgari, S., & Rivers, D. B. (2011). Venom proteins from endoparasitoid wasps and their role in host-parasite interactions. *Annual review of entomology*, *56*, 313-335.

Bale, J. S., Van Lenteren, J. C., & Bigler, F. (2008). Biological control and sustainable food production. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1492), 761-776.

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *cell*, *136*(2), 215-233.

Beck, M., Theopold, U., & Schmidt, O. (2000). Evidence for serine protease inhibitor activity in the ovarian calyx fluid of the endoparasitoid Venturia canescens. *Journal of Insect Physiology*, *46*(9), 1275-1283.

Beckage, N. E., & Gelman, D. B. (2004). Wasp parasitoid disruption of host development: implications for new biologically based strategies for insect control. *Annual review of entomology*, *49*, 299.

Berezikov, E. (2011). Evolution of microRNA diversity and regulation in animals. *Nature Reviews Genetics*, *12*(12), 846-860.

Bergman, Å., Heindel, J. J., Jobling, S., Kidd, K., Zoeller, T. R., & World Health Organization. (2013). *State of the science of endocrine disrupting chemicals 2012*. World Health Organization.

Bravo, A., Gill, S. S., & Soberon, M. (2007). Mode of action of Bacillus thuringiensis Cry and Cyt toxins and their potential for insect control. *Toxicon*, *49*(4), 423-435.

Brodeur, J., & Boivin, G. (2004). Functional ecology ot immature parasitoids. *Annu Rev Entomol*, *49*, 27-9.

Carthew, R. W., & Sontheimer, E. J. (2009). Origins and mechanisms of miRNAs and siRNAs. *Cell*, *136*(4), 642-655.

Casartelli, M., Corti, P., Leonardi, M. G., Fiandra, L., Burlini, N., Pennacchio, F., & Giordana, B. (2005). Absorption of albumin by the midgut of a lepidopteran larva. *Journal of insect physiology*, *51*(8), 933-940.

Casartelli, M., Cermenati, G., Rodighiero, S., Pennacchio, F., & Giordana, B. (2008). A megalin-like receptor is involved in protein endocytosis in the midgut of an insect (Bombyx mori, Lepidoptera). *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *295*(4), R1290-R1300.

Christiaens, O., Niu, J., & Nji Tizi Taning, C. (2020). RNAi in insects: a revolution in fundamental research and pest control applications. *Insects*, *11*(7), 415.

Colinet, D., Kremmer, L., Lemauf, S., Rebuf, C., Gatti, J. L., & Poirié, M. (2014). Development of RNAi in a Drosophila endoparasitoid wasp and demonstration of its efficiency in impairing venom protein production. *Journal of insect physiology*, *63*, 56-61.

Communication from the Commission to the European Parliament, the European Council, the Council, the European Economic and Social Committee and the Committee of the Regions, The European Green Deal, COM(2019) 640 final.

Cuny, M. A., & Poelman, E. H. (2022). Evolution of koinobiont parasitoid host regulation and consequences for indirect plant defence. *Evolutionary Ecology*, 1-21.

Dahlman, D. L. (1990). Evaluation of teratocyte functions: an overview. *Archives of Insect Biochemistry and Physiology*, *13*(3-4), 159-166.

Dara, S. K. (2019). The new integrated pest management paradigm for the modern age. *Journal of Integrated Pest Management*, *10*(1), 12.

Deguine, J. P., Aubertot, J. N., Flor, R. J., Lescourret, F., Wyckhuys, K. A., & Ratnadass, A. (2021). Integrated pest management: good intentions, hard realities. A review. *Agronomy for Sustainable Development*, *41*(3), 1-35.

Dhakal, R., & Singh, D. N. (2019). Biopesticides: a key to sustainable agriculture. *Int J Pure App Biosci*, *7*(3), 391-396.

Dykxhoorn, D. M., & Lieberman, J. (2005). The silent revolution: RNA interference as basic biology, research tool, and therapeutic. *Annual review of medicine*, *56*, 401.

Ellers, J., & Jervis, M. (2003). Body size and the timing of egg production in parasitoid wasps. *Oikos*, *102*(1), 164–172. https://doi.org/10.1034/j.1600-0706.2003.12285.x

Falabella, P., Tremblay, E., & Pennacchio, F. (2000). Host regulation by the aphid parasitoid Aphidius ervi: the role of teratocytes. *Entomologia Experimentalis et Applicata*, *97*(1), 1-9.

FAO (2020) NSP – Integrated Pest Management, FAO definition. http://www.fao.org/agriculture/crops/thematic-sitemap/theme/pests/ipm/en/.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *nature*, *391*(6669), 806-811.

Fitches, E., Woodhouse, S. D., Edwards, J. P., & Gatehouse, J. A. (2001). In vitro and in vivo binding of snowdrop (Galanthus nivalis agglutinin; GNA) and jackbean (Canavalia ensiformis; Con A) lectins within tomato moth (Lacanobia oleracea) larvae; mechanisms of insecticidal action. *Journal of Insect Physiology*, *47*(7), 777-787.

Fitches, E., Audsley, N., Gatehouse, J. A., & Edwards, J. P. (2002). Fusion proteins containing neuropeptides as novel insect contol agents: snowdrop lectin delivers fused allatostatin to insect haemolymph following oral ingestion. *Insect Biochemistry and Molecular Biology*, *32*(12), 1653-1661.

Fitches, E., Edwards, M. G., Mee, C., Grishin, E., Gatehouse, A. M. R., Edwards, J. P., & Gatehouse, J. A. (2004). Fusion proteins containing insect-specific toxins as pest control agents: Snowdrop lectin delivers fused insecticidal spider venom toxin to insect haemolymph following oral ingestion. *Journal of Insect Physiology*, *50*(1), 61–71.

Flint, M. L., & Van den Bosch, R. (2012). Introduction to integrated pest management. Springer Science & Business Media.

Godfray, H. C. J., & Godfray, H. C. J. (1994). *Parasitoids: behavioral and evolutionary ecology* (Vol. 67). Princeton University Press.

Gómez, I. C., Sääksjärvi, I. E., Mayhew, P. J., Pollet, M., Rey del Castillo, C., Nieves-Aldrey, J. L., ... & Tuomisto, H. (2018). Variation in the species

richness of parasitoid wasps (Ichneumonidae: Pimplinae and Rhyssinae) across sites on different continents. *Insect Conservation and Diversity*, *11*(3), 305-316.

Hajek, A. E., & Eilenberg, J. (2018). *Natural enemies: an introduction to biological control*. Cambridge University Press.

Harvey, J. A., & Malcicka, M. (2016). Nutritional integration between insect hosts and koinobiont parasitoids in an evolutionary framework. *Entomologia Experimentalis et Applicata*, *159*(2), 181–188. https://doi.org/10.1111/eea.12426

Henri, D. C., & Van Veen, F. F. (2011). Body size, life history and the structure of host–parasitoid networks. In *Advances in ecological research* (Vol. 45, pp. 135-180). Academic Press.

Jeffers, L. A., & Roe, R. M. (2008). The movement of proteins across the insect and tick digestive system. *Journal of insect physiology*, *54*(2), 319-332.

Jeffers, L. A., Shen, H., Khalil, S., Bissinger, B. W., Brandt, A., Gunnoe, T. B., & Roe, R. M. (2012). Enhanced activity of an insecticidal protein, trypsin modulating oostatic factor (TMOF), through conjugation with aliphatic polyethylene glycol. *Pest management science*, *68*(1), 49-59.

Jeffers, L. A., Shen, H., Bissinger, B. W., Khalil, S., Gunnoe, T. B., & Roe, R. M. (2014). Polymers for the stabilization and delivery of proteins topically and per os to the insect hemocoel through conjugation with aliphatic polyethylene glycol. *Pesticide biochemistry and physiology*, *115*, 58-66.

Kachhawa, D. (2017). Microorganisms as a biopesticides. *J Entomol Zool Stud*, *5*(3), 468-473.

Kennerdell, J. R., & Carthew, R. W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell*, *95*(7), 1017-1026.

Kim, K. H., Kabir, E., & Jahan, S. A. (2017). Exposure to pesticides and the associated human health effects. *Science of the total environment*, *575*, 525-535.

Kumari, P., Jasrotia, P., Kumar, D., Kashyap, P. L., Kumar, S., Mishra, C. N., ... & Singh, G. P. (2022). Biotechnological Approaches for Host Plant Resistance to Insect Pests. *Frontiers in Genetics*, *13*.

Lundgren, J. G., & Duan, J. J. (2013). RNAi-based insecticidal crops: potential effects on nontarget species. *Bioscience*, *63*(8), 657-665.

Mahmood I, Imadi SR, Shazadi K, Gul A and Hakeem KR (2016). Effects of pesticides on environment. In: *Plant, soil and microbes* 1:253–269.

Mackauer, M., & Sequeira, R. (1993). Patterns of Development in Insect Parasites. In *Parasites and Pathogens of Insects* (pp. 1–23). Elsevier. https://doi.org/10.1016/B978-0-08-091649-1.50006-2

Miresmailli, S., & Isman, M. B. (2014). Botanical insecticides inspired by plant–herbivore chemical interactions. *Trends in plant science*, *19*(1), 29-35.

Moreau, S. J., & Asgari, S. (2015). Venom proteins from parasitoid wasps and their biological functions. *Toxins*, *7*(7), 2385-2412.

Oerke, E. C. (2006). Crop losses to pests. *The Journal of Agricultural Science*, *144*(1), 31-43.

Pedata, P. A., Garonna, A. P., Zabatta, A., Zeppa, P., Romani, R., & Isidoro, N. (2003). Development and morphology of teratocytes in Encarsia berlesei and Encarsia citrina: first record for Chalcidoidea. *Journal of Insect Physiology*, *49*(11), 1063-1071.

Pennacchio, F., & Strand, M. R. (2006). Evolution of developmental strategies in parasitic Hymenoptera. *Annu. Rev. Entomol.*, *51*, 233-258.

Pennacchio, F., & Mancini, D., (2012). Aphid parasitoid venom and its role in host regulation. In Parasitoid viruses: symbionts and pathogens. Edited by BeckageNE, DrezenJ-M. Elsevier pages: 247–254.

Pennacchio, F., Caccia, S., & Digilio, M. C. (2014). Host regulation and nutritional exploitation by parasitic wasps. *Current Opinion in Insect Science*, *6*, 74-79.

Pérez-García, A., Romero, D., & De Vicente, A. (2011). Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. *Current opinion in biotechnology*, *22*(2), 187-193.

Persley, G. J. (2000). *Agricultural Biotechnology and the Poor: Promethean Science*. Washington, D.C.: Consultative Group on International Agricultural Research (CGIAR), 3–21.

Peterson, R. K., Higley, L. G., & Pedigo, L. P. (2018). Whatever happened to IPM?. American Entomologist, 64(3), 146-150.

Pimentel, D., & Burgess, M. (2014). Environmental and economic costs of the application of pesticides primarily in the United States. In *Integrated pest management* (pp. 47-71). Springer, Dordrecht.

Poirié, M., Carton, Y., & Dubuffet, A. (2009). Virulence strategies in parasitoid Hymenoptera as an example of adaptive diversity. *Comptes rendus biologies*, *332*(2-3), 311-320.

Poirié, M., Colinet, D., & Gatti, J. L. (2014). Insights into function and evolution of parasitoid wasp venoms. *Current Opinion in Insect Science*, *6*, 52-60.

Roh, J. Y., Choi, J. Y., Li, M. S., Jin, B. R., & Je, Y. H. (2007). Bacillus thuringiensis as a specific, safe, and effective tool for insect pest control. *Journal of microbiology and biotechnology*, *17*(4), 547-559.

Scott, J. G., Michel, K., Bartholomay, L. C., Siegfried, B. D., Hunter, W. B., Smagghe, G., ... & Douglas, A. E. (2013). Towards the elements of successful insect RNAi. *Journal of insect physiology*, *59*(12), 1212-1221.

Smith, J. J., Herzig, V., King, G. F., & Alewood, P. F. (2013). The insecticidal potential of venom peptides. *Cellular and Molecular Life Sciences*, *70*(19), 3665-3693.

Sparks, T. C., Crouse, G. D., & Durst, G. (2001). Natural products as insecticides: the biology, biochemistry and quantitative structure–activity relationships of spinosyns and spinosoids. *Pest management science*, *57*(10), 896-905.

Stehle, S., & Schulz, R. (2015). Agricultural insecticides threaten surface waters at the global scale. *Proceedings of the National Academy of Sciences*, *112*(18), 5750-5755.

Stenberg, J. A., Sundh, I., Becher, P. G., Björkman, C., Dubey, M., Egan, P. A., ... & Viketoft, M. (2021). When is it biological control? A framework of definitions, mechanisms, and classifications. *Journal of Pest Science*, *94*(3), 665-676.

Strand, M. R. (2014). Teratocytes and their functions in parasitoids. *Current Opinion in Insect Science*, *6*, 68-73.

Strand, M. R., & Burke, G. R. (2015). Polydnaviruses: from discovery to current insights. *Virology*, *479*, 393-402.

Thakur, N., Kaur, S., Tomar, P., Thakur, S., & Yadav, A. N. (2020). Microbial biopesticides: current status and advancement for sustainable agriculture and environment. In *New and future developments in microbial biotechnology and bioengineering* (pp. 243-282). Elsevier.

Xue, X. Y., Mao, Y. B., Tao, X. Y., Huang, Y. P., & Chen, X. Y. (2012). New approaches to agricultural insect pest control based on RNA interference. *Advances in insect physiology*, *42*, 73-117.

Vinson, S. B., & work (s):, G. F. I. R. (1980). Host Regulation by Insect Parasitoids. *The Quarterly Review of Biology*, *55*(2), 143–165.

Vinson, S. B. (1990). How parasitoids deal with the immune system of their host: an overview. *Archives of insect biochemistry and physiology*, *13*(1-2), 3-27.

Wajnberg, E., & Colazza, S. (Eds.). (2013). *Chemical ecology of insect parasitoids*. John Wiley-Blackwell, London.

Wang, B., Ren, C., Yang, L., Fang, Q., Song, Q., & Ye, G. (2020). Venom α-amylase of the endoparasitic wasp Pteromalus puparum influences host metabolism. *Pest Management Science*, *76*(6), 2180–2189.

Wang, Z. Z., Ye, X. Q., Shi, M., Li, F., Wang, Z. H., Zhou, Y. N., ... & Chen, X. X. (2018). Parasitic insect-derived miRNAs modulate host development. *Nature communications*, *9*(1), 1-9.

Wightwick, A., Walters, R., Allinson, G., Reichman, S., & Menzies, N. (2010). Environmental risks of fungicides used in horticultural production systems. *Fungicides*, *1*, 273-304.

Yu, D. S., Chen, Y. B., Li, M., Yang, M. J., Yang, Y., Hu, J. S., & Luo, K. J. (2016). A polydnaviral genome of Microplitis bicoloratus bracovirus and molecular interactions between the host and virus involved in NF-κB signaling. *Archives of virology*, *161*(11), 3095-3124.

2 *In vivo* characterization of venom *γ*-glutamyl-transpeptidase of the endoparasitic wasp *Aphidius ervi*

The first part of this PhD thesis focuses on the functional characterization of the most abundant protein of *Aphidius ervi* venom protein, a γ-glutamyl-transpeptidase (*Ae*-γ-GT). The text has been submitted for publication in Journal of Insect Physiology.

2.1 Introduction

The association *Acyrthosiphon pisum* (Homoptera: Aphididae)-*Aphidius ervi* (Hymenoptera: Braconidae) is a well-established model system to study the physiology of host-parasitoid interactions (Pennacchio et al 1999; Rahbé et al. 2002; Pennacchio et al., 2012). *A. ervi* is a solitary koinobiont endoparasitoid of various Macrosiphinae aphid species, whose immature stages develop within the host body and emerge as adults from the mummified aphids (Starý, 1970).

One of the most evident symptoms observed in parasitized aphids is the reduction or even the suppression of reproduction, which has been reported in many host parasitoid associations (Polaszek, 1986; Brown and Kainoh, 1992; Digilio et al., 2000; Beckage and Gelman, 2004). This degenerative effect of parasitism on ovaries can be observed as early as 24 h after parasitoid oviposition, before egg hatching, suggesting that molecules injected during oviposition are responsible for this observed alteration (Polaszek, 1986). Indeed, the host regulation process is driven by maternal factors, such as venom and, at a later stage of development, by teratocytes, cells deriving from the dissociation of the embryonic membrane which *de novo* synthesize and secrete a number of molecules (Pennacchio et al., 2014).

The venom of parasitic Hymenoptera is a complex mixture of proteinaceous and non-proteinaceous compounds injected by adult wasps into the host during parasitization, along with the egg (Moreau and Asgari, 2015). *A. ervi* venom is composed of several proteins, among which γ-glutamyl transpeptidase (Ae-γ-GT), an enzyme involved in glutathione metabolism, is by far the most abundant component, responsible for host castration (Digilio et al. 1998; 2000; Falabella et al., 2007). Colinet et al. (2014a) identified two different sequences of *γ-GT* in *A. ervi* venom: *Ae-γ-GT1* (=Contig2) and *Ae-γ-*

GT2 (=Contig6), which are likely products of two genes that originated from a duplication event (i.e., paralogs) (Dennis et al., 2020). *Ae*-γ-GT targets the upper part of the host ovarioles, inducing the apoptotic degeneration of the germaria and the young apical embryos (Digilio et al., 2000; Falabella et al., 2007). This limits the development of new embryos, allowing the redirection of the host metabolic effort in support of its reproduction in favour of the development of the wasp larva (Pennacchio et al., 1995; 1999; Caccia et al., 2012), which is further reinforced by the action of teratocytes. Indeed, these cells, freely floating in the host haemocoel, are engaged in a form of extra-oral digestion of host reproductive tissues, releasing back into the haemolymph complex nutrients in a processed form that can be used by the developing parasitoid larvae (Falabella et al. 2000; Pennacchio and Mancini, 2012). Moreover, bacteriocytes, which remain unaltered in parasitized hosts and harbour a higher load of primary symbionts, contribute to the increased host nutritional suitability for the developing wasp progeny (Pennacchio et al., 1999; Falabella et al., 2000; 2005; 2009; Rahbé et al., 2002). In addition to the direct action of parasitoid venom on the pea aphid ovaries, host exploitation is favored by a network of interactions among teratocytes and the host primary endosymbiont *Buchnera aphidicola* (Falabella et al., 2000, 2009; Pennacchio and Mancini, 2012),; this is characterized by an increased rate of symbiont-mediated biosynthesis of essential amino acids (Cloutier and Douglas, 2003; Rahbé et al., 2002) and by the production of parasitism-specific proteins by the teratocytes, playing an important role in the host regulation process and growth of the parasitoid juveniles (Falabella et al., 2005; 2009).

Regulation of the intricate network of interactions in this tripartite system (parasitoid, host and its primary symbiont) is far from being understood from a functional point of view, and the role of host regulation factors in the modulation of host metabolic balance affecting parasitoid growth and development remains elusive. This is even more complex in koinobiont parasitoids, where the surviving host represents an “open” system in which the nutritional resources are made available by a finely-controlled process of metabolic redirection (Vinson and Iwantsch, 1980; Mackauer et al., 1997; Pennacchio et al., 2014). Therefore, the regulation of host metabolism can have a significant impact on parasitoid fitness, since the developmental conditions experienced by early life stages may play a significant role in the variation of adult life-history traits (Jervis, 2008), including longevity and fecundity (Henri and Van Veen, 2011; Harvey and Malcicka, 2016).

Here we focus on this aspect of host regulation, considering the possible role of the most abundant venom component, *Ae*-γ-GT. The functional role of *Ae*-γ-GT has already been studied by injection experiments of purified protein (Digilio et al., 1998; 2000; Falabella et al., 2007). This approach, however, has been limited to the use of protein doses that are not physiological and, even more importantly, in the absence of other venom components that could have complementary and interactive roles in the modulation of host regulation mechanisms.
To overcome this limitation, here we have developed a reverse genetics approach, based on *Ae-γ-GT* silencing by RNA interference (RNAi) in *A. ervi* females. The phenotypic changes associated with gene silencing of both parasitized host aphids and wasp progeny were assessed, enabling a new role for this abundant venom protein in host regulation mediated by *A. ervi* venom, to be described. The proposed method provides a new approach for investigating *in vivo* the role of specific venom components in braconid aphid parasitoids.

2.2 Materials and methods

2.2.1 Insect rearing

A. pisum was reared on *Vicia faba* (broad bean) and kept in an environmental chamber, at 20 ± 1°C, 75 ± 5% relative humidity, under a photoperiod of 16:8 h (L:D). *A. ervi* was reared on *A. pisum*, maintained on potted broad bean plants and kept in a separate climate room and exposed at 25 ± 1°C, under photoperiod and humidity conditions as described above. Aphids and parasitoids cultures were started with insect material originally collected on alfalfa plants, in Eboli (Salerno province), Southern Italy, and refreshed periodically with field collected material.

2.2.2 RNA extraction from *A. ervi*

Total RNA was extracted from whole *A. ervi* females and venom glands using TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -80°C according to the manufacturer's instructions. The concentration and purity of total RNA were determined by measuring the 260/280 nm absorbance ratio with a Varioskan Flash (Thermo Fisher Scientific).

2.2.3 DNA extraction from *A. pisum*

DNA was extracted from *A. pisum* according to Holmes and Bonner (1973). Briefly, aphids were homogenized in 200 µL of lysis buffer (2 % sodium dodecyl sulfate, 7 M urea, 0.35 M NaCl, 1 mM EDTA, and 0.01 M Tris, pH 8). An equal volume of phenol solution was added, and samples were shaken for 15 min at RT (room temperature). The phases were separated by centrifugation at 10,000 × g for 10 min at RT. The aqueous phase was collected in a new tube and 2.5 volume of 96% ethanol, and 0.1 volume of 3M sodium acetate (pH 5) were added to precipitate the DNA. The concentration and purity of total DNA was evaluated by assessing 260/280 nm absorbance ratio using Varioskan Flash (Thermo Fisher Scientific).

2.2.4 Synthesis of dsRNA targeting *Ae-γ-GT*

Primers flanked by T7 promoter sequences (Table 2.1) were designed to amplify a 442 bp fragment targeting both *Ae-γ-GT1* and *Ae-γ-GT2*. Briefly, total RNA extracted from *A. ervi* venom gland was retrotranscribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. From the first PCR reactions 2 µL of the obtained template were used. We obtained a 567 bp *Ae-γ-GT* cDNA fragment, which was used as a template for a nested PCR reaction with T7-flanking primers. All primers are listed in Table 2.1. Reaction mixes were prepared using Phusion High-Fidelity DNA Polymerase 2 U/µL (Thermo Fisher Scientific), according to manufacturer's instructions, purified with PureLink PCR Purification Kit (Thermo Fisher Scientific), eluted in nuclease-free water, and quantified measuring the absorbance as described above. The resulting T7-flanking purified PCR product acted as a template to synthesize dsRNA directed against *Ae-γ-GTs* (ds-γGT), using the MEGAscript® RNAi kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Then, dsRNA preparations were quantified by measuring their absorbance as desc
ribed above and run on 1% agarose gel to confirm their integrity. A GFP dsRNA (ds-GFP), used in control experiments, was similarly produced starting from the cloning vector pcDNA 3.1/CT-GFP TOPO (Thermo Fisher Scientific), which was used as template for a PCR reaction, performed as described elsewhere (Caccia et al, 2020). The obtained dsRNA was stored in the elution solution at -20°C.

2.2.5 dsRNA microinjection in *A. ervi* females

The dsRNA was administered via microinjection into day-1 *A. ervi* female pupae, at the onset of the reddish pigmentation of their compound eyes (Figure 2.1), since at this stage the venom apparatus is not fully developed (MacGrill, 1924) and, thus, venom secretion is not yet started or very limited. Newly formed *A. pisum* mummies containing pre-pupae, as indicated by the larval meconium presence, were selected and opened 24-36 h later, when the pupal stage described above was attained. Briefly, these mummies were stuck ventrally onto a Petri dish cover, using double-sided sticky tape, gently opened using dissecting needles and, then, observed to check the red pigmentation of the eyes (Figure 2.2A).

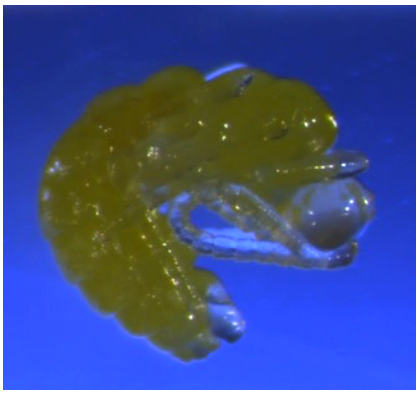

Fig. 2.1 Day-1 *Aphidius ervi* female pupa without reddish pigmentation of its compound eyes. This stage was selected as the most suitable for dsRNA injections.

The dsRNA was injected into the hemocoel of these female pupae, between the last two abdominal segments (Figure 2.2B), under a stereomicroscope (SteREO Discovery.V8, Zeiss, Jena, Germany). A volume of 60 nl of ds-γGT solution was injected, at a concentration of 4,2 ng/μL (~250 ng/pupa), using a FemtoJet® 4 × microinjection device (Eppendorf, AG, Hamburg, Germany) and capillary needles (1.0 mm × 0.75 mm glass pipettes with a 7-9mm taper, a 0.8-0.5 μm tip, and a 40-80 MΩ of resistance) drawn out on a Sutter P-97 laser micropipette puller (Sutter Instrument®, Novato Ca, USA) with the following program: LINE = 1, Heat = 535, PULL = 80, VEL= 70, DEL= 80, PRESS = 200. The injection conditions were set to 100 hPa injection pressure (pi), 10 hPa compensation pressure (pc) and 0.7 s injection time (t). A ds-GFP solution was used as off-target dsRNA and administered to a group of *A. ervi* control pupae.

A. ervi female pupae that received an injection were kept in Petri dishes, under the same environmental conditions indicated above, until adult emergence. To assess the time window of gene silencing, virgin females were collected from day 1 to day 5 after emergence and stored at -80°C until RNA extraction and quantitative PCR (qRT-PCR) analysis.

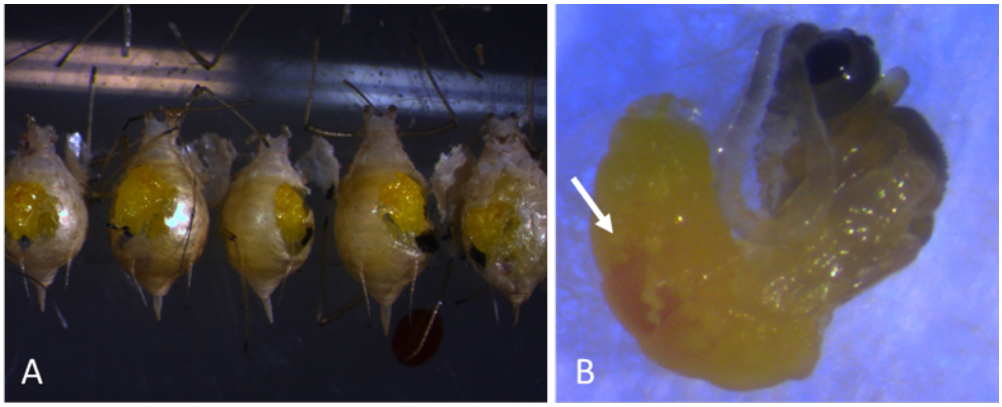

Figure 2.2 Mummies of *Acyrthosiphon pisum* adhered on double-sided tape that have been dissected to reveal the parasitoid's pupal stages (A) and microinjection trials in *Aphidius ervi* pupae (B). The arrow points to the injection site, where a PBS/dye solution was injected.

2.2.6 Controlled parasitization

After emergence, *A. ervi* females were isolated in Petri dishes and allowed to mate with a single male of the same age. Couples were fed ad libitum with a 50% honey-water solution (v/v) for about 6 h, and, after 12 h used for parasitization as hereafter described. Third instar *A. pisum* nymphs were singly introduced in a Petri dish with an *A. ervi* female that received an injection as pupa of *Ae-γ-GT*-dsRNA, or of GFP dsRNA, for controls. After being stung two times by the parasitoid wasp, aphids were considered parasitized. All aphids parasitized by the same *A. ervi* female were isolated on a *V. fava* plant, separated from the rest of the population, while female wasps were immediately processed for RNA extraction to perform qRT-PCR analysis and confirm the effective silencing of *Ae-γ-GT1* and *Ae-γ-GT2* genes.

2.2.7 qRT-PCR analysis

Differential relative expression of *Ae-γ-GT1* and *Ae-γ-GT2* was assessed by means of SYBR Green qRT-PCR, using the primer pairs

reported in Table 2.1, to check gene silencing efficiency. All primers were designed using Primer Express, version 1.0 software (Applied Biosystems, Carlsbad, CA, USA). mRNA levels were measured by one-step qRT-PCR using the SYBR Green PCR Kit (Applied Biosystems) and 1 µL of RNA template (100 ng), according to the manufacturer's instructions. Relative gene expression data were analyzed using the $2^{\Delta\Delta CT}$ method. For validation of the ΔΔCt method the difference between the Ct value of *Ae-γ-GTs* and the Ct value of the *A. ervi* ribosomal protein *28Sβ2* transcripts [ΔCt = Ct (*Ae-γ-GTs*) - Ct (*28 Sβ2*)] was plotted versus the log of two-fold serial dilutions (200, 100, 50, 25 and 12.5 ng) of the purified RNA samples. The plot of log total RNA input versus ΔCt displayed a slope lower than 0.1, indicating that the efficiencies of the two amplicons were approximately equal.

2.2.8 Host and wasp changes induced by *Ae-γ-GT* gene silencing

We assessed the impact of *Ae-γ-GTs* gene silencing (γGT^-) on parasitized *A. pisum* and on wasp's progeny, using as controls non-parasitized aphids (NP) and intact wasps with a complete venom blend (γGT^+), respectively. The body size of experimental aphids was assessed under a stereomicroscope (SteREO Discovery V8, Zeiss), measuring 48 h (before parasitoid egg hatching) and 5 days after parasitization (when 3rd instar larvae and large teratocytes are present) the area delimited by the body outline. On this latter sampling time, aphids were dissected in ice-cold 4% paraformaldehyde in PBS (phosphate buffered saline 1×; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4), under a stereomicroscope SteREO Discovery V8, (Zeiss). Upon dissection, the size of parasitoid larvae and teratocytes was assessed, measuring the area delimited by the larval body outline and outer cell membrane, respectively. Castration effect of parasitism was assessed by counting the total number of embryos and measuring the size of the 3 largest embryos (stage 18 and higher, as defined in Miura et al., 2003). All measurement were performed using the image analysis software ZEN (Zeiss).

The adults emerging from aphids parasitized by silenced (γGT^-) and control (γGT^+) females were compared assessing longevity and body size of both sexes, while fecundity was determined on mated females. Adult survival was daily checked on wasps singly isolated in glass vials (75 × 10 mm), provided with a 50% honey-water solution (v/v) and kept under the same environmental conditions reported above.

The lifetime fecundity (LF) of γGT$^+$ and γGT$^-$ wasps was determined on 10 mated females, scoring the daily number of parasitized aphids over the entire life (Mayhew, 2016; Watt et al., 2016). One day after emergence, females were mated, then placed in a transparent plastic container (30 x 30 x 15 cm) and daily provided with an infested *V. faba* plant, bearing at least 100 *A. pisum* third instar nymphs, to prevent superparasitism. The survival of each ovipositing female and the total number of parasitized aphids (i.e., mummies produced) were daily recorded. Briefly, females were dissected in a drop of PBS by 2 h after their emergence and the number of eggs per female was counted. For each wasp, the metatibia length was measured as a proxy of body size (Ellers and Jervis, 2003). All experiments were conducted under the environmental conditions described above for insect rearing.

2.2.9 Absolute quantification of *B. aphidicola* by qPCR

qPCR experiments were carried out to evaluate the bacterial load of *B. aphidicola* in *A. pisum* 4th instar nymphs at 12 and 48 h after parasitization. Absolute abundance was determined using qPCR targeting *dnaK* gene of *B. aphidicola* and normalized to the number of the host *rpl7* gene copies, as described elsewhere (Kešnerová et al., 2017).

qPCR reactions were performed using the SYBR Green PCR Kit (Applied Biosystems), according to the manufacturer's instructions. All primers (Table 2.1) were designed using Primer Express, version 1.0 software (Applied Biosystems). Raw *B. aphidicola* genome copy number of each sample was determined by interpolating the Ct value of the *dnaK* gene with the standard curve obtained using a known quantity of the targeted DNA. Standard curve was performed on ten-fold serial dilution of the purified PCR amplicon encoding the *dnaK* gene (5, 0.5, 0.05, 0.005 and 0.0005 ng). The *dnaK* copy number was calculated based on the molecular weight and the DNA concentration of the purified amplicon. Raw abundances were normalized by dividing their values by the number of *rpl7* gene copies present in the sample, which was determined using the same procedure. This normalized value of *dnaK* gene copies was then multiplied by the median number of *rpl7* gene copies of the samples of a given dataset, to obtain normalized *B. aphidicola* genome equivalents per 100 ng of total DNA.

Primers	Sequence 5' to 3'	Melt. temp. (°C)	Lenght (bp)	Gene
Ae-γ-GT1 Fw *Ae-γ-GT1* Rw	GTTGATGTAGATGTTCAAGA ATTGGATCCTTGAACACTT	54	567	*Ae-γ-GT*1
Ae-γ-GT T7 Fw *Ae-γ-GT* T7 Rw	TAATACGACTCACTATAGGGAG GGTGTTGTCAGTGAATCAACT TAATACGACTCACTATAGGGAG AGCACAAATGGCACTGGTGA	70	442	*Ae-γ-GT1* and *Ae-γ-GT*2
q*Ae-γ-GT1* Fw q*Ae-γ-GT1* Rw	CATTATCATCAATGGCTCCTTC GTTTCGACCACAAGTATCTAG	60	132	*Ae-γ-GT1*
q*Ae-γ-GT2* Fw q*Ae-γ-GT2* Rw	CTCCTGTTCCTCCAATAACAACT ATCTTGAGTTTCTTCGCCTCCAG	68	126	*Ae-γ-GT2*
q28Sβ2 Fw q28Sβ2 Rw	GCTCAGCGAGGACAGAAAC TCGATAGGCCGTGTTTCGC	60	100	28S Subunit β2
qDnaK Fw qDnaK Rw	GCTGCAGTACAGGGAGGA GATGTCATTATTCCACCCATA	58	107	*B. aphidicola dnaK*
qRpl7 Fw qRpl7 Rw	TTGAAGAGCGTAAGGGAACTG TATTGGTGATTGGAATGCGTT	58	76	*A. pisum rpl7*

Table 2.1 Primers used for *Ae-γ-GTs* expression analysis and *Buchnera aphidicola* quantification. Underlined sequences indicate T7 promoters.

2.3 Statistical Analysis

The assumption of normal distribution of data was tested and met by Shapiro-Wilk test. Each dataset was checked for homoscedasticity using Levene's test. One-Way ANOVA test followed by Tukey's post-hoc test was used to compare aphid size and ovary size in the different experimental aphid groups. Kruskal-Wallis test followed by Dunn's pairwise test was used to compare bacterial load in aphids. Mann Whitney test was used to analyze th*e Ae-γ-GT1* gene expression in microinjected parasitoids. Student's t-test was used to compare *Ae-γ-GT2* expression*,* morphometric parameters of larvae, teratocytes and adult parasitoids. All data were analyzed using Prism software, v8.0 (GraphPad software; San Diego, California, USA).

2.4 Results

2.4.1 *Ae-γ-GT* time course analysis

The *Ae-γ-GT1* gene was significantly silenced at all time points, from 1 to 5 days post-emergence ($P<0.0001$), as shown in Figure 2.3. When used for parasitization experiments, to evaluate the effect of gene silencing on host regulation and the development of progeny, female wasps displayed a high level of parasitization activity and a 100% survival rate on day 2 post-emergence (30 hours).

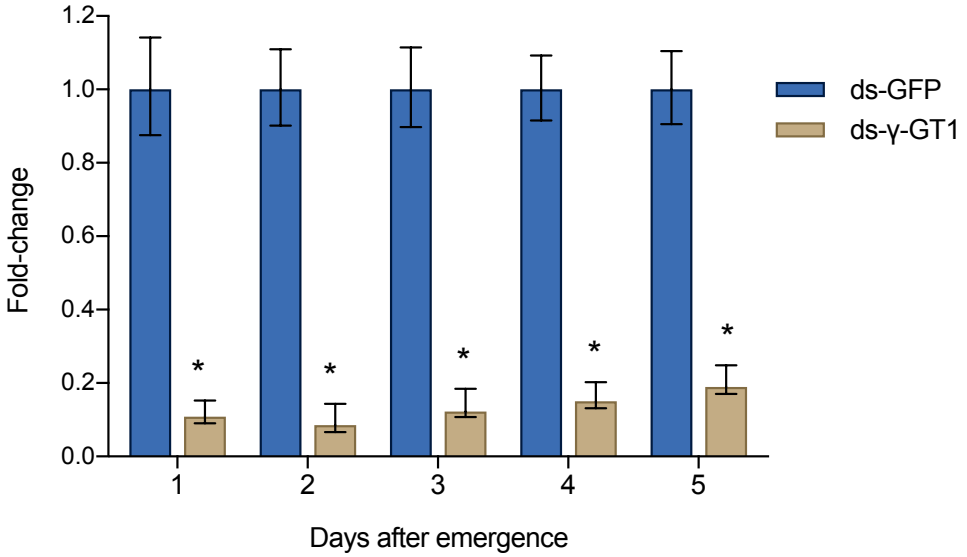

Fig. 2.3 Time course analysis of *Ae-γ-GT1* transcription level after the injection of dsRNA targeting this gene. Significant differences (Student's t-test: $P < 0.0001$; see Table 3.1) withing each sampling time are denoted with an asterisk. Details of statistical analysis are available in Table 2.2.

Time	Comparison	t	df	P value
Day 1	ds-GFP - ds-γGT	11.45	21	< 0,0001
Day 2	ds-GFP - ds-γGT	5.281	14	< 0,0001
Day 3	ds-GFP - ds-γGT	10.01	29	< 0,0001
Day 4	ds-GFP - ds-γGT	8.248	12	< 0,0001
Day 5	ds-GFP - ds-γGT	7.33	19	< 0,0001

Table 2.2 Student's t-test comparison of ΔCt values in the time course experiment on *Ae-γGT1* silencing.

2.4.2 *Ae-γ-GT1* and *Ae-γ-GT2* relative expression

The experimental females receiving an injection of dsRNA showed transcription rates, both for *Ae-γ-GT1* and *Ae-γ-GT2,* significantly reduced compared to controls (P<0.0001) (Fig. 2.4).

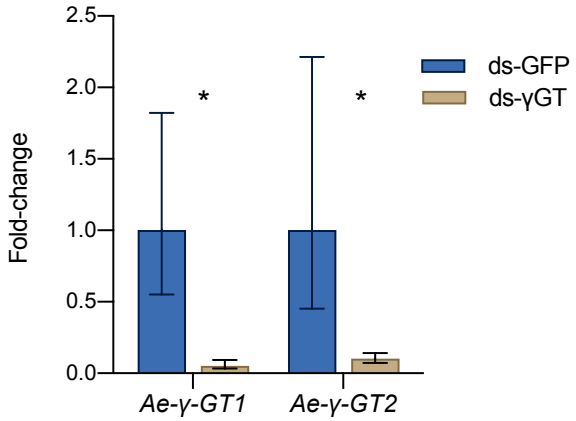

Fig. 2.4. Relative expression of *Ae-γ-GT1* (Mann Whitney test, U = 39, *P* = 0.0032; n_1 = 14, n_2 = 15) and *Ae-γ-GT2* (Student's *t*-test: *P* = 0.0015, t = 3.773, df = 17) in 30 hour-old females receiving an injection of dsRNA targeting both genes (mean ± SE)**.** Mean values denoted with an asterisk are significantly different.

2.4.3 Phenotypic changes in *A. pisum*

As shown in Figure 2.5 and 2.6, aphids that were parasitized by γGT$^-$ wasps were significantly larger (P < 0.0001) on both day 2 and day 5 after oviposition compared to those that were parasitized by γGT$^+$ females, which did not differ from non-parasitized controls (NP).

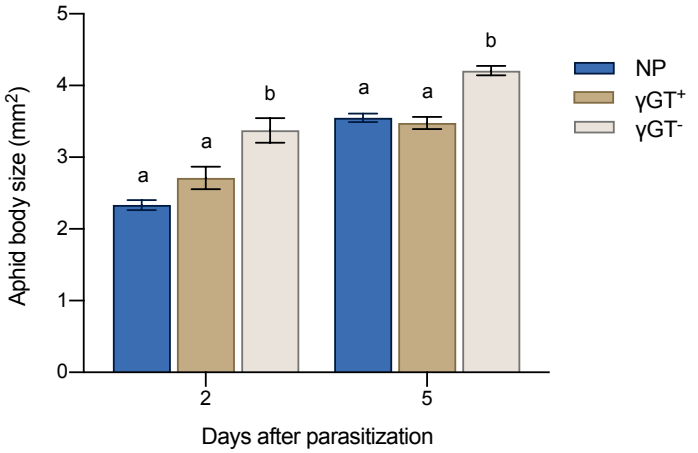

Fig. 2.5 Size of parasitized aphids as affected by *Ae-γ-GT* gene silencing. On day 2 after parasitoid oviposition, aphids parasitized by *Ae-γ-GT* silenced *Aphidius ervi* (γGT$^-$) were larger in size than non-parasitized controls (NP) and aphids parasitized by *A. ervi* that received an injection of GFP-dsRNA (γGT$^+$) (One-way ANOVA: $F_{(2, 48)}$ = 14.32; *P* < 0.0001), which did not significantly differ between them. This difference persisted at day 5 (One-way ANOVA: $F_{(2, 44)}$ = 32.35; *P* < 0.0001). Bars show the mean ± SE; mean values denoted with different letters are significantly different.

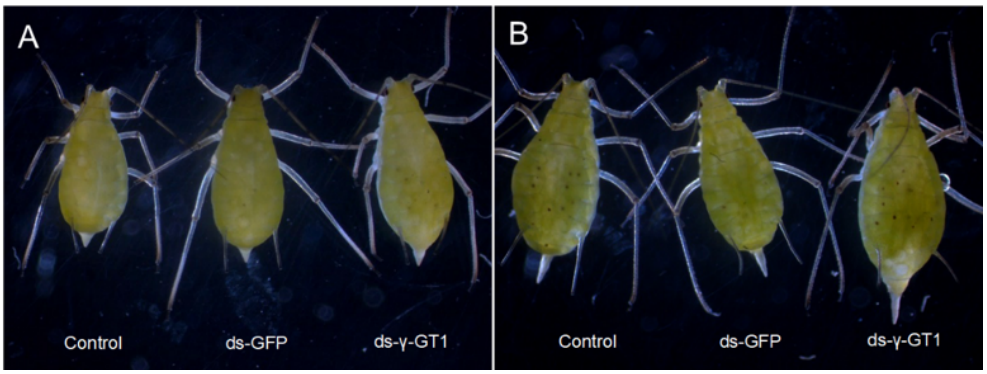

Fig. 2.6 Aphid size comparisons at 48 hours (A) and 5 days (B) following parasitization as affected by *Ae-γ-GT* gene silencing.

On day 5, for each experimental group, aphids were dissected and aphid ovaries, parasitoid larvae and teratocytes were collected. The number of embryos resulted significantly higher in NP and γGT$^-$ aphids, compared to γGT$^+$ aphids ($P < 0.0001$) (Fig. 2.7A; 2.8). The same pattern was observed also for the size of the largest embryos, which resulted significantly ($P < 0.0001$) larger in aphids parasitized by γGT$^-$ wasps than in those exposed to γGT$^+$ wasps, while no differences were observed between NP and γGT$^-$ experimental groups (Fig. 2.7B; 2.8).

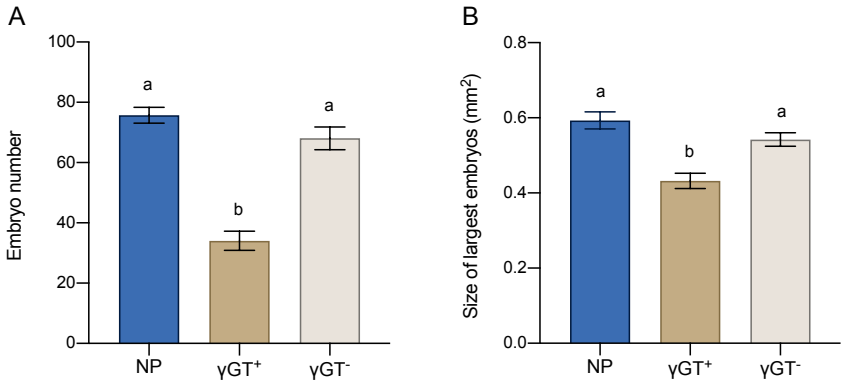

Fig. 2.7 Number and size alteration of aphid embryos in parasitized aphids as affected by *Ae-γ-GTs* gene silencing. (A) The number of embryos in dissected aphids on the 5th day after parasitization resulted higher in NP and γGT$^-$ aphids, compared to γGT$^+$ aphids (One-way ANOVA: $F_{(2, 42)} = 47.8$; $P < 0.0001$). (B) The size of the largest embryos resulted greater in γGT$^-$ and NP, than in γGT$^+$ (One-way ANOVA: $F_{(2, 50)} = 15.88$; $P < 0.0001$), while did not differ in NP and γGT$^-$ aphids. Bars show the mean ± SE; mean values denoted with different letters are significantly different.

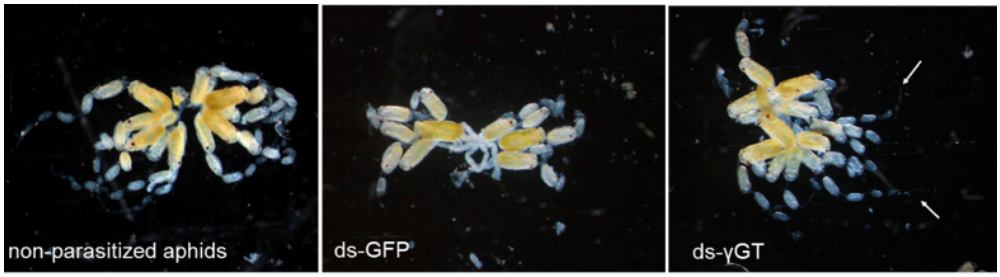

Fig. 2.8 Phenotypic comparison of dissected ovaries in *Acyrthosiphon pisum* between the three experimental groups as affected by *Ae-γ-GT* gene silencing. The presence of still intact germaria in the aphids parasitized by *Ae-γ*-GT silenced females are indicated by the arrows.

2.4.4 Size of *A. ervi* larvae and teratocytes

Parasitoid larvae and teratocytes collected from γGT$^-$ parasitized aphids were significantly (P < 0.0001) larger than those from γGT$^+$ aphids (Fig ure 2.9; 2.10).

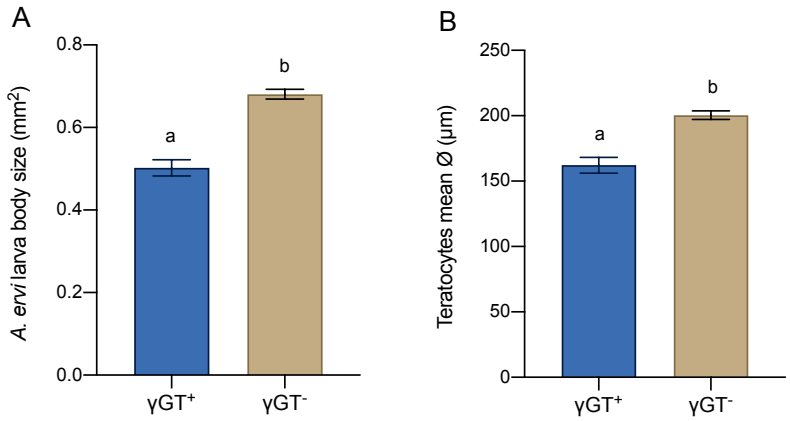

Fig. 2.9 Size of *Aphidius ervi* larvae and teratocytes as affected by *Ae-γ-GTs* gene silencing. Larvae (A) and teratocytes (B) were collected from aphids dissected 5 days post-parasitization by *A. ervi* females that received as pupae an injection of *Ae-γ-GT*-dsRNA (γGT$^-$) or of GFP-dsRNA (γGT$^+$). Both larvae (Student's *t*-test: P < 0.0001, t =7.786, df = 28) and teratocytes diameter (Student's *t*-test: P < 0.0001, t = 5.557, df = 28) were significantly larger in γ-GT$^-$, compared to γGT$^+$ wasp females. Bars show the mean ± SE; mean values denoted with different letters are significantly different.

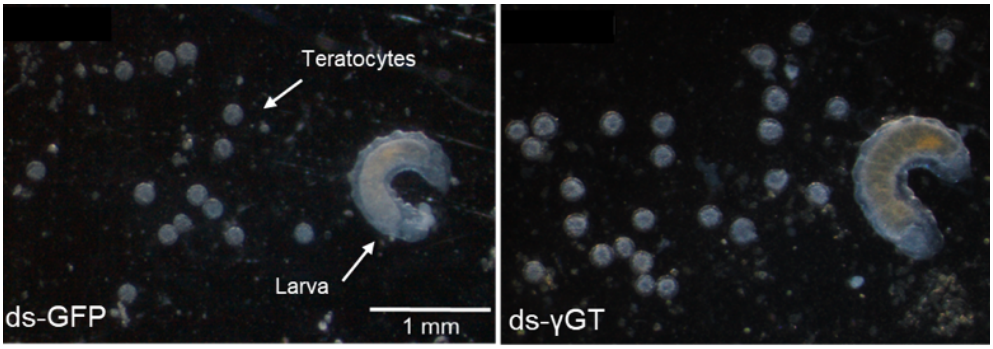

Fig. 2.10 Size comparisons of *Aphidius ervi* larvae and teratocytes explanted from aphids parasitized by wasps treated with ds-GFP and ds-γGT.

2.4.5 *Buchnera* load

Because the bacterial primary symbiont *B. aphidicola* provides a substantial metabolic support to this complex interaction between the host aphid and the developing parasitoid larva (Pennacchio et al. 1999), we decided to assess if the *Ae-γ-GT* gene silencing had any impact on the number of genome copies of *B. aphidicola* in parasitized host aphids. Bacterial load was significantly ($P < 0.0001$) higher in aphids parasitized by γGT$^-$ *A. ervi* females than in aphids parasitized by γGT$^+$ wasps, which were higher but did not significantly differ from non-parasitized controls (NP), both at 12 and 48 h after parasitization (Figure 2.11).

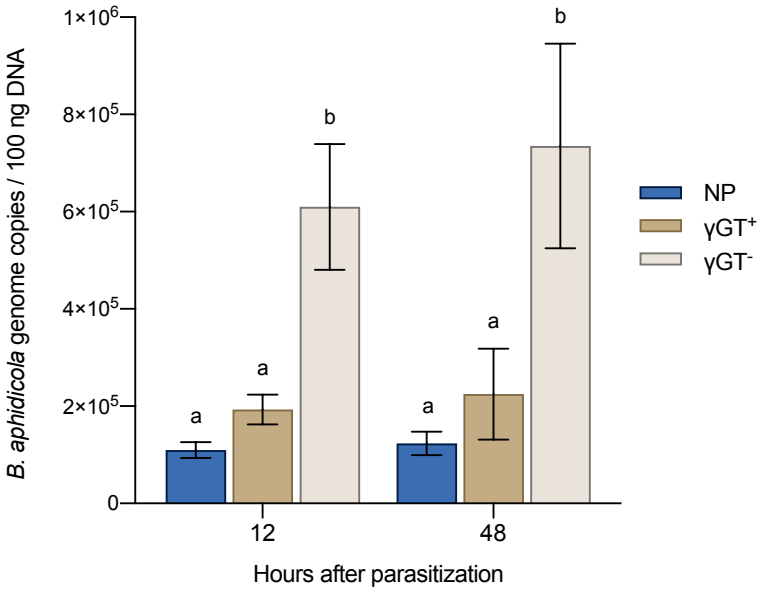

Fig. 2.11 *Buchnera aphidicola* load in parasitized aphids as affected by *Ae-γ-GT* gene silencing. *B. aphidicola* load in aphids parasitized by *A. ervi* silenced for *Ae-γ-GT* (γGT$^-$) was significantly higher than in non-parasitized (NP) and parasitized controls (γGT$^+$), both at 12 h (Kruskal-Wallis; $P < 0.0001$; K-W statistic = 22.94) and 48 h (Kruskal-Wallis; $P < 0.0001$; K-W statistic = 22.01) after parasitization. Bars show the mean ± SE; mean values denoted with different letters are significantly different.

2.4.6 Effects on *A. ervi* adult progeny

The size increase of the wasp progeny induced by *Ae-γ-GT* gene silencing was consistently observed also in adults, which were significantly larger (P = 0.0004 for females and P = 0.0164 for males) when generated by γGT$^-$ wasps (Table 2.3). However, this size increase was associated with an inverse pattern of longevity, which was significantly lower in the larger virgin adults (P = 0.0361 for females and P = 0.0015 for males) and in ovipositing females (P = 0.0232) emerging from aphids parasitized by γGT$^-$ wasps (Table 1). The *Ae-γ-GT* gene silencing was also associated with a nearly 50% reduction of the number of mature eggs at the emergence (P = 0.0006) and a significant reduction of lifetime fecundity (P = 0.0282) (Table 2.2).

Parameter	Category	γGT$^-$ (n)	γ-GT$^+$ (n)	*P* value
Size of mummy (mm^2)	Both sexes	3.57 ± 0.08 (31)	3.59 ± 0.08 (28)	0.8299
Adult longevity (days)	Virgin males*	4.8 ± 0.15 (29)	5.4 ± 0.10 (25)	0.0015
	Virgin females*	7.8 ± 0.29 (18)	8.7 ± 0.26 (17)	0.0361
Metatibia length (mm)	Virgin males*	0.87 ± 0.006 (29)	0.84 ± 0.01(25)	0.0164
	Virgin females*	0.97 ± 0.01 (18)	0.91 ± 0.009 (17)	0.0004
Developmental time (egg to adult) (days)	Virgin males	14.8 ± 0.13 (29)	14.6 ± 0.21 (25)	0.3585
	Virgin females	15.06 ± 0.22 (18)	15.06 ± 0.26 (17)	0.9924
Adult longevity (days)	Ovipositing females*	11.5 ± 0.93 (10)	14.3 ± 0.63 (10)	0.0232
Mature eggs (n)	Emerged females	48.8 ± 7.67 (10)	88.1 ± 5.56 (10)	0.0006
Lifetime fecundity (n° mummies)	Ovipositing females*	242.6± 24.73 (10)	315.1± 17.64 (10)	0.0282

Table 2.3 Biological and morphometric traits of the progeny produced by *Aphidius ervi* females as affected by *Ae-γ-GT* gene silencing. Size of adult wasps generated by *A. ervi* females with a venom lacking *Ae*-γ-GT was larger than in controls, while longevity and reproduction were both negatively affected. Reported values are means ± SE, and significant differences (Student's t-test: $P < 0.05$; see Table 2.4) between the two experimental groups are denoted with an asterisk.

Parameter	Specimen	T[1]	Df[2]	*P* value
Size of mummy (mm^2)	Both sexes	0.2158	57	0.8299
Adult longevity (days)	Virgin males*	3.355	52	0.0015
	Virgin females*	2.185	33	0.0361
Metatibia length (mm)	Virgin males*	2.480	52	0.0164
	Virgin females*	3,985	33	0.0004
Developmental time (egg to adult) (days)	Virgin males	0.9264	52	0.3585
	Virgin females	0.009	33	0.9924
Adult longevity (days)	Ovipositing females*	2.481	18	0.0232
Mature eggs (n)	Emerging females*	4.145	18	0.0006
Lifetime fecundity (n° mummies)	Ovipositing females*	2.387	18	0.0282

Table 2.4. Student's t-test comparison of life-history traits reported in Table 1. [1] T-score; [2] Degrees of freedom.

2.5 Discussion

Molecules produced by parasitoids are potential candidates for the development of innovative biotechnologies for insect control. The exploitation of these molecules in sustainable strategies of pest control is largely dependent on an in-depth knowledge of their molecular and functional characteristics (Pennacchio et al., 2012).

The venom of parasitic wasps is a complex cocktail of molecules injected into the host along with egg by the ovipositing female, which regulate host physiology to circumvent the immune barriers and meet the demanding nutritional needs of the developing parasitoid's juveniles (Pennacchio and Strand, 2006). These venoms have been extensively studied from a functional and molecular point of view, revealing a great diversity of roles in the host regulation and subsequent exploitation strategies (Asgari and Rivers, 2011; Becchimanzi et al., 2020; Colinet et al., 2014a; Danneels et al., 2010; Sim and Wheeler, 2016).

Here we present a novel approach to study the role of specific venom components in aphid-parasitoid interactions, based on RNAi-mediated gene knockdown, using the well-established model *A. ervi* - *A. pisum*. In this model system, *Ae*-γ-GT is the most abundant component of *A. ervi* venom, which is known to induce host castration and to redirect the nutritional resources supporting *A. pisum* reproduction in favour of the development of the wasp's progeny (Falabella et al., 2007; Pennacchio and Mancini, 2012). However, it is unlikely that the major component of a venom blend has only one function. To assess

whether *Ae*-γ-GT plays additional roles in the regulation of aphid physiology, we generated *Ae*-γ-GT deficient parasitoids by RNAi.
RNAi-mediated gene silencing has been shown to be effective in reducing the expression of venom components at both the transcript and protein level (Colinet et al., 2014b; Martinson et al., 2016; Siebert et al., 2015). In line with these studies, we found that *Ae*-γ-GT mRNA levels at adult emergence are significantly reduced by dsRNA injection into pupal stages of *A. ervi*.
Based on the high abundance of *Ae*-γ-GT in *A. ervi* venom, we expected a marked alteration of parasitism success as a result of knocking down the encoding gene. However, this was not the case, since this gene silencing was associated with unaltered rates of parasitism success and, surprisingly, to an even better growth performance of the progeny laid by wasps delivering a γGT^- venom blend. Moreover, this was preceded by an increase in aphid growth, starting on day 2 after parasitoid oviposition, before egg hatching. On day 2, the parasitoid egg is not yet hatched, and the venom is the main host regulation factor in action. At this stage, the larger size of aphids is associated with a higher abundance of the primary symbiont *B. aphidicola*, known to provide metabolic support to aphid reproduction (Douglas, 1998; Pennacchio and Mancini, 2012; Skaljac, 2016), which proliferates more in hosts receiving an injection of venom with reduced content of *Ae*-γ-GT. This provides indirect evidence of an unexpected role of *Ae*-γ-GT as a metabolic pacemaker which controls *Buchnera* proliferation, likely induced by other venom components.
Furthermore, knockdown of the *Ae-γ-GT* gene prevented ovarian degeneration, which in contrast was observed in GFP control wasps, where the number of embryos and their size in hosts parasitized by these latter wasps were similar to those recorded in non-parasitized aphids. This finding is in full agreement with previous studies demonstrating the host castration effect by *A. ervi* parasitism (Polaszek, 1986), induced by venom (Digilio et al., 2000), and specifically mediated by *Ae*-γ-GT (Falabella et al., 2007). Our results provide direct experimental evidence, under *in vivo* physiological conditions, which supports the negative impact of *Ae*-γ-GT on host reproductive tissues.
In naturally parasitized host aphids, their castration is mediated by *Ae*-γ-GT, which triggers apoptosis in the upper part of the ovarioles, preventing the formation of new embryos (Digilio et al., 2000; Falabella et al., 2007), while those already formed are later digested by teratocytes, releasing nutrients in a suitable form for the parasitoid

larvae, in correspondence of their most intense growth phase (Pennacchio et al., 1995, 1999; Pennacchio and Mancini, 2012). Therefore, the early boost of *Buchnera* and the associated higher growth of aphids and their ovaries, induced by *Ae-γ-GT* silencing, provided the parasitoid progeny with a more abundant and diverse food source, which determined the significantly larger size of wasp larvae and teratocytes. This scenario of nutritional enrichment by *Ae*-γ-GT silencing results in higher growth rates of developing larvae and adults, which, however, were less viable and showed a reduced reproductive performance.

The size-dependent fitness paradigm largely applying to *A. ervi* (He and Wang, 2006) and, in general, to parasitic Hymenoptera (Honěk, 1993; Jervis et al., 2003; Arakawa et al., 2004; Gao et al., 2016) is in contrast with our data. However, the larger size attained by *A. ervi* in hosts offering a much richer food substrate is the result of an abnormal nutritional regimen rather than the expression of a genetic potential. Diet composition has an evident impact on physiology and lifespan (Simpson and Raubenheimer, 2007; Simpson et al., 2017). Research on diet-dependent development in the model fruit fly *Drosophila* has clearly shown that dietary manipulation induces metabolic abnormalities both in larvae and adults (Baker and Thummel, 2007; Bass et al., 2007; Padmanabha and Baker, 2014). For example, diet composition has been shown to affect metabolic homeostasis in fruit flies, as a high-carbohydrate diet leads to fat accumulation and weight gain (Gáliková and Klepsatel, 2018; van Dam et al., 2020). The protein-to-carbohydrate ratio of the fly diet significantly affects adult lifespan and suppresses their fertility (Morris et al., 2012; van Dam et al., 2020). Therefore, we can reasonably assume that the altered nutritional suitability of the host resulting from *Ae-γ-GT* silencing is responsible for an altered profile of metabolism and growth of wasp larvae which causes a fitness cost in the emerging adults.

In conclusion, we have identified a new function of *A. ervi* venom, which finely regulates the complex interaction among the parasitoid larva, the host, and its primary symbiont. The modulation of the metabolic contribution of the primary symbiont appears to be of crucial importance for an adequate regulation of host nutritional suitability. Basically, the enhancement of symbiont metabolic contribution to the whole system, induced by unidentified venom components, is influenced by *Ae*-γ-GT. Altering this compensatory effect leads to an altered growth pattern and reduced fitness. This is a particularly interesting phenomenon, that shows how a long-lasting and complex

regulation of host physiology can be triggered by the venom blend through a multifaceted conditioning of the host's primary symbionts, finely modulating the intricate network of nutritional interactions underlying the optimal developmental pattern, that maximizes the fitness of the wasp.

Our results reveal a further layer of complexity in host regulation by *A. ervi*, which partly accounts for the observed variability of host suitability observed when different host instars are parasitized (Sequeira and Mackauer, 1992; Mackauer, 1993; Mackauer et al., 1997). Our findings further corroborate the importance of basic nutritional studies for promoting the success of *in vitro* rearing technologies for parasitic Hymenoptera (Grenier, 2009).

Moreover, the use of recombinant *Ae*-γ-GT protein can facilitate further characterizations *in vivo* and *in vitro*, which may aid in the development of this very interesting venom component as a biopesticide molecule.

References

Arakawa, R., Miura, M. and Fujita, M., (2004). Effects of host species on the body size, fecundity, and longevity of *Trissolcus mitsukurii* (Hymenoptera: Scelionidae), a solitary egg parasitoid of stink bugs. *Applied Entomology and Zoology*, *39*(1), pp.177-181.

Asgari, S., and Rivers, D.B. (2011). Venom proteins from endoparasitoid wasps and their role in host-parasite interactions. Annu. Rev. Entomol. *56*, 313–335.

Baker, K. D., & Thummel, C. S. (2007). Diabetic larvae and obese flies—emerging studies of metabolism in *Drosophila*. *Cell metabolism*, *6*(4), 257-266.

Bass, T. M., Grandison, R. C., Wong, R., Martinez, P., Partridge, L., & Piper, M. D. (2007). Optimization of dietary restriction protocols in *Drosophila*. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *62*(10), 1071-1081.

Becchimanzi, A., Avolio, M., Bostan, H., Colantuono, C., Cozzolino, F., Mancini, D., Chiusano, M.L., Pucci, P., Caccia, S., and Pennacchio, F. (2020). Venomics of the ectoparasitoid wasp *Bracon nigricans*. BMC Genomics *21*, 34.

Beckage, N. E., & Gelman, D. B. (2004). Wasp parasitoid disruption of host development: implications for new biologically based strategies for insect control. *Annual review of entomology*, *49*, 299.

Brown JJ, Kainoh Y. 1992. Host castration by *Ascogaster* spp. (Hymenoptera: Braconidae). Ann. Entomol. Soc. Am. 85: 67–71

Caccia, S., Grimaldi, A., Casartelli, M., Falabella, P., de Eguileor, M., Pennacchio, F., and Giordana, B. (2012). Functional analysis of a fatty acid binding protein produced by *Aphidius ervi* teratocytes. J. Insect Physiol. *58*, 621–627.

Caccia, S., Astarita, F., Barra, E., Di Lelio, I., Varricchio, P., & Pennacchio, F. (2020). Enhancement of *Bacillus thuringiensis* toxicity by feeding *Spodoptera littoralis* larvae with bacteria expressing immune suppressive dsRNA. *Journal of Pest Science*, *93*(1), 303-314.

Cloutier, C., and Douglas, A.E. (2003). Impact of a parasitoid on the bacterial symbiosis of its aphid host. Entomol. Exp. Appl. *109*, 13–19.

Colinet, D., Anselme, C., Deleury, E., Mancini, D., Poulain, J., Azéma-Dossat, C., Belghazi, M., Tares, S., Pennacchio, F., Poirié, M., et al. (2014a). Identification of the main venom protein components of *Aphidius ervi*, a parasitoid wasp of the aphid model *Acyrthosiphon pisum*. BMC Genomics *15*, 342.

Colinet, D., Kremmer, L., Lemauf, S., Rebuf, C., Gatti, J.-L., and Poirié, M. (2014b). Development of RNAi in a *Drosophila* endoparasitoid wasp and demonstration of its efficiency in impairing venom protein production. J. Insect Physiol. *63*, 56–61.

Danneels, E.L., Rivers, D.B., and de Graaf, D.C. (2010). Venom proteins of the parasitoid wasp *Nasonia vitripennis*: recent discovery of an untapped pharmacopee. Toxins *2*, 494–516.

Dennis, A.B., Ballesteros, G.I., Robin, S., Schrader, L., Bast, J., Berghöfer, J., Beukeboom, L.W., Belghazi, M., Bretaudeau, A., Buellesbach, J., et al. (2020). Functional insights from the GC-poor genomes of two aphid parasitoids, Aphidius ervi and *Lysiphlebus fabarum*. BMC Genomics *21*, 376.

Digilio, M.C., Isidoro, N., Tremblay, E., and Pennacchio, F. (2000). Host castration by *Aphidius ervi* venom proteins. J. Insect Physiol. *46*, 1041–1050.

Douglas, A.E. (1998). Nutritional Interactions in Insect-Microbial Symbioses: Aphids and Their Symbiotic Bacteria *Buchnera*. Annu. Rev. Entomol. *43*, 17–37.

Ellers, J., and Jervis, M. (2003). Body size and the timing of egg production in parasitoid wasps. Oikos *102*, 164–172.

Falabella, P., Tremblay, E., and Pennacchio, F. (2000). Host regulation by the aphid parasitoid *Aphidius ervi*: the role of teratocytes. Entomol. Exp. Appl. *97*, 1–9.

Falabella, P., Riviello, L., Caccialupi, P., Rossodivita, T., Teresa Valente, M., Luisa De Stradis, M., Tranfaglia, A., Varricchio, P., Gigliotti, S., Graziani, F., et al. (2007). A gamma-glutamyl transpeptidase of *Aphidius ervi* venom induces apoptosis in the ovaries of host aphids. Insect Biochem. Mol. Biol. *37*, 453–465.

Falabella, P., Riviello, L., De Stradis, M.L., Stigliano, C., Varricchio, P., Grimaldi, A., de Eguileor, M., Graziani, F., Gigliotti, S., and Pennacchio, F. (2009). *Aphidius ervi* teratocytes release an extracellular enolase. Insect Biochem. Mol. Biol. *39*, 801–813.

Gáliková, M., & Klepsatel, P. (2018). Obesity and aging in the *Drosophila* model. *International journal of molecular sciences*, *19*(7), 1896.

Grenier, S. (2009). In vitro rearing of entomophagous insects—past and future trends: a minireview. *Bull. Insectol*, *62*, 1-6.

Harvey, J. A., & Malcicka, M. (2016). Nutritional integration between insect hosts and koinobiont parasitoids in an evolutionary framework. *Entomologia experimentalis et applicata*, *159*(2), 181-188.

He, X.Z., and Wang, Q. (2006). Asymmetric size effect of sexes on reproductive fitness in an aphid parasitoid *Aphidius ervi* (Hymenoptera: Aphidiidae). Biol. Control *36*, 293–298.

Henri, D. C., & Van Veen, F. F. (2011). Body size, life history and the structure of host–parasitoid networks. In *Advances in ecological research* (Vol. 45, pp. 135-180). Academic Press.

Holmes, D.S., and Bonner, J. (1973). Preparation, molecular weight, base composition, and secondary structure of giant nuclear ribonucleic acid. Biochemistry *12*, 2330–2338.

Honěk, A. (1993). Intraspecific variation in body size and fecundity in insects: A general relationship. Oikos 66, 483–492.

Jervis, M. A., Ferns, P. N., & Heimpel, G. E. (2003). Body size and the timing of egg production in parasitoid wasps: a comparative analysis. *Functional Ecology*, *17*(3), 375-383.

Jervis, M. A., Ellers, J., & Harvey, J. A. (2008). Resource acquisition, allocation, and utilization in parasitoid reproductive strategies. *Annual review of entomology*, *53*(1), 361-385.

Kešnerová, L., Mars, R.A.T., Ellegaard, K.M., Troilo, M., Sauer, U., and Engel, P. (2017). Disentangling metabolic functions of bacteria in the honey bee gut. PLOS Biol. *15*, e2003467.

MacGrill, E.I. (1924). IV.—The Life-History of *Aphidius avenæ* (Hal.), a Braconid parasitic on the Nettle Aphis (*Macrosiphum urticæ*). Proc. R. Soc. Edinb. *43*, 51–71.

Mackauer, M. (1993). Patterns of development in insect parasites. *Parasites and pathogens of insects*, *1*, 1-23.

Mackauer, M., Sequeira, R. & Otto, M. (1997) Growth and development in parasitoid wasps: adaptation to variable host resources. Vertical Food Web Interactions (eds K.Dettmer, G. Baur & W. Volkl), pp. 191–203. Springer-Verlag. Berlin.

Martinson, E.O., Martinson, V.G., Edwards, R., Mrinalini, null, and Werren, J.H. (2016). Laterally transferred gene recruited as a venom in parasitoid wasps. Mol. Biol. Evol. *33*, 1042–1052.

Mayhew, P.J. (2016). Comparing parasitoid life histories. Entomol. Exp. Appl. *159*, 147–162.

Miura, T., Braendle, C., Shingleton, A., Sisk, G., Kambhampati, S. and Stern, D.L. (2003), A comparison of parthenogenetic and sexual embryogenesis of the pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphidoidea). J. Exp. Zool., 295B: 59-81. https://doi.org/10.1002/jez.b.3

Moreau, S.J.M., and Asgari, S. (2015). Venom proteins from parasitoid wasps and their biological functions. Toxins *7*, 2385–2412.

Morris, S. N. S., Coogan, C., Chamseddin, K., Fernandez-Kim, S. O., Kolli, S., Keller, J. N., & Bauer, J. H. (2012). Development of diet-induced insulin resistance in adult *Drosophila melanogaster*. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1822*(8), 1230-1237.

Padmanabha, D., & Baker, K. D. (2014). *Drosophila* gains traction as a repurposed tool to investigate metabolism. *Trends in Endocrinology & Metabolism*, *25*(10), 518-527.

Pennacchio, F., and Mancini, D. (2012). Chapter 20 - Aphid Parasitoid Venom and its Role in Host Regulation. In Parasitoid Viruses, Nancy E. Beckage, and Jean-Michel Drezen, eds. (San Diego: Academic Press), pp. 247–254.

Pennacchio, F., and Strand, M.R. (2006). Evolution of developmental strategies in parasitic hymenoptera. Annu. Rev. Entomol. *51*, 233–258.

Pennacchio, F., Digilio, M.C., and Tremblay, E. (1995). Biochemical and metabolic alterations in *Acyrthosiphon pisum* parasitized by Aphidius ervi. Arch. Insect Biochem. Physiol. *30*, 351–367.

Pennacchio, F., Fanti, P., Falabella, P., Digilio, M.C., Bisaccia, F., and Tremblay, E. (1999). Development and nutrition of the braconid wasp, *Aphidius ervi* in aposymbiotic host aphids. Arch. Insect Biochem. Physiol. *40*, 53–63.

Pennacchio, F., Giordana, B., and Rao, R. (2012). Chapter 22 - Applications of Parasitoid Virus and Venom Research in Agriculture. In Parasitoid Viruses, Nancy E. Beckage, and Jean-Michel Drezen, eds. (San Diego: Academic Press), pp. 269–283.

Pennacchio, F., Caccia, S., and Digilio, M.C. (2014). Host regulation and nutritional exploitation by parasitic wasps. Curr. Opin. Insect Sci. *6*, 74–79.

Polaszek, A. (1986). The effects of two species of hymenopterous parasitoid on the reproductive system of the pea aphid, *Acyrthosiphon pisum*. *Entomologia experimentalis et applicata*, *40*(3), 285-292.

Rahbé, Y., Digilio, M.C., Febvay, G., Guillaud, J., Fanti, P., and Pennacchio, F. (2002). Metabolic and symbiotic interactions in amino acid pools of the pea aphid, *Acyrthosiphon pisum*, parasitized by the braconid *Aphidius ervi*. J. Insect Physiol. *48*, 507–516.

Skaljac, M. (2016). Chapter 5 - Bacterial symbionts of aphids (Hemiptera: Aphididae). In Biology and ecology of Aphids, Andreas Vilcinskas, ed. (CRC Pess) pp. 100-125.

Starý P (1970) *Biology of Aphid Parasites (Hymenoptera: Aphidiidae) with Respect to Integrated Control*. Dr. W. Junk N.V., The Hague, The Netherlands.Sequeira, R., and Mackauer, M. (1992). Nutritional Ecology of an Insect Host-Parasitoid Association: The Pea Aphid-*Aphidius Ervi* System. Ecology *73*, 183–189.

Siebert, A.L., Wheeler, D., and Werren, J.H. (2015). A new approach for investigating venom function applied to venom calreticulin in a parasitoid wasp. Toxicon Off. J. Int. Soc. Toxinology *107*, 304–316.

Sim, A.D., and Wheeler, D. (2016). The venom gland transcriptome of the parasitoid wasp *Nasonia vitripennis* highlights the importance of novel genes in venom function. BMC Genomics *17*, 571.

Simpson, S. J., & Raubenheimer, D. (2007). Caloric restriction and aging revisited: the need for a geometric analysis of the nutritional bases of aging. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *62*(7), 707-713.

Simpson, S. J., Le Couteur, D. G., Raubenheimer, D., Solon-Biet, S. M., Cooney, G. J., Cogger, V. C., & Fontana, L. (2017). Dietary protein, aging and nutritional geometry. *Ageing research reviews*, *39*, 78-86.

van Dam, E., van Leeuwen, L. A., Dos Santos, E., James, J., Best, L., Lennicke, C., ... & Cochemé, H. M. (2020). Sugar-induced obesity and insulin resistance are uncoupled from shortened survival in *Drosophila*. *Cell metabolism*, *31*(4), 710-725.

Vinson, S. B., & Iwantsch, G. F. (1980). Host regulation by insect parasitoids. *The Quarterly Review of Biology*, *55*(2), 143-165.

Watt, T.J., Duan, J.J., Tallamy, D.W., Hough-Goldstein, J., Ilvento, T.W., Yue, X., and Ren, H. (2016). Reproductive and developmental biology of the emerald ash borer parasitoid *Spathius galinae* (Hymenoptera: Braconidae) as affected by temperature. Biol. Control *96*, 1–7.

3 Effects on survival and ultrastructural anomalies induced by recombinant *Tn*BVANK1 in *Acyrthosiphon pisum*

3.1 Introduction

The growing demand for sustainable and environmentally friendly pest control methods has led to the exploration of protein-based approaches (Edwards and Gatehouse, 2007; Hesham et al., 2021), such as the use of insecticidal protein toxins from *Bacillus thuringensis* (Bt) (Karabörklü et al., 2018). The diverse array of molecules produced by entomophagous insects to exploit their victims provides an additional rich source of natural compounds with potential insecticide activity (Pennacchio et al., 2012). In particular, the highly diverse group of parasitic Hymenoptera represents a nearly untapped source of bioinsecticides worth of intensive research efforts (Pennacchio and Strand, 2006; Poirié et al., 2014; Pennacchio et al., 2012). Parasitic wasps display a wide range of host-exploitation strategies (Vinson and Iwantsch, 1980; Pennacchio et al., 2014), some of which involve the use of venom (idiobionts) to rapidly and irreversibly paralyze their hosts (Pennacchio and Strand, 2006) or to regulate multiple physiological traits (koinobionts) (Cuny et al., 2022). To subdue their victims, females of koinobiont endoparasitic wasps inject several regulation factors at oviposition that alter host physiology, such as venom proteins and, in some cases, symbiotic viruses which encode potent virulence factors (Pennacchio and Strand, 2006; Pennacchio et al., 2014).

Polydnaviruses (PDVs) are unique mutualistic viruses found in many species of koinobiont parasitoids in the superfamily Ichneumonoidea that parasitize larval stages of Lepidoptera (Volkoff et al., 2012). PDVs are among the most potent viral immunosuppressors existing in nature and encode host regulation factors which are able to modulate host physiology (Beckage, 1998; Pennacchio and Strand 2006; Di Lelio et al., 2014), by disrupting its vital functions and providing a suitable nutritional environment to the parasitoid larvae (Strand and Burke, 2014). Viral particles are produced by specialized cells from the calyx region of the wasp ovaries (Volkoff et al., 2012) and stored in the lumen of the lateral oviducts until their release into the host hemocoel during parasitization (Webb et al., 2000; Beckage and Gelman, 2004).

This part of the thesis work focuses on the insecticidal activity of a virulence factor encoded by the polydnavirus associated with the

braconid wasp *Toxoneuron nigriceps* (*Tn*BV) (Falabella et al., 2003). This virulence factor, known as *TnBVANK1*, is a member of the viral ankyrin (ANK) protein family (Falabella et al., 2006; Salvia et al., 2017; Valzania et al., 2014) and appears involved in both immunosuppressive and endocrine alterations of its host (Strand, 2012; Di Lelio et al., 2014). While these molecules are typically expected to act on receptors located in the haemocoel of the host (Pennacchio et al., 2012), previous studies have shown that PDVs-encoded proteins are also active when orally administered (Maiti et al., 2003; Gill et al., 2006). In particular, Di Lelio et al., 2014, found that *Tn*BVANK1 had insecticidal activity towards *Spodoptera littoralis* larvae, when fed with transgenic tobacco plants expressing the viral gene.

The present study aims at investigating the insecticidal effects of the *Tn*BVANK1 against the pea aphid (*Acyrthosiphon pisum*), following oral ingestion. The development of new biotechnologies to control aphids and, more in general, sap-sucking pests, is limited by the lack of effective natural molecules that can be used. Here, we contribute to this research gap, by assessing and characterizing the insecticide activity of *Tn*BVANK1 on the pea aphid *Acyrthosiphon pisum*.

Recombinant protein engineering technology was employed to obtain the biologically active *Tn*BVANK1 protein using the yeast *Pichia pastoris* as expression system. Artificial feeding bioassays were used to assess the oral toxicity of *Tn*BVANK1 on aphids, while a preliminary evaluation of its effect was carried out by microscopy observations on the gut tissues.

3.2 Materials and Methods

3.2.1 Production of recombinant *Tn*BVANK1

The recombinant *Tn*BVANK1 protein was synthesized and generously provided by Morena Casartelli of the University of Milan. Briefly, *Tn*BVANK1 was generated using the commercially available pGAPZαA vector, which contained the coding sequence for the ank1 protein (accession number AJ583457) with a 6X-His tag at the C-terminal end for identification and purification (Figure 3.1). The protein was expressed in the wild type strain X-33 of *P. pastoris*, which was used as a heterologous expression system for large scale fermentation.

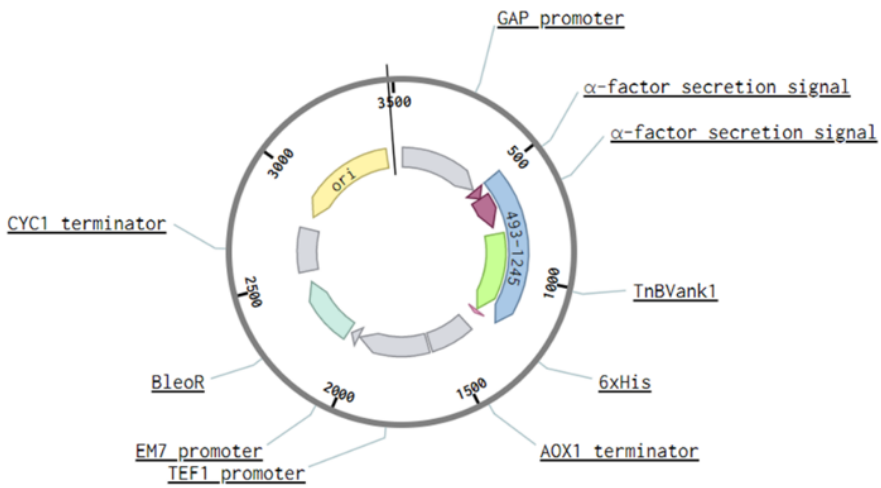

Fig. 3.1 pGAPZαA plasmid design, which utilizes the GAP promoter to consistently express recombinant proteins in *Pichia pastoris*. The estimated size of the recombinant *Tn*BVANK1 produced is approximately 17 kDa.

3.2.2 Artificial diet bioassays

The toxicity of *Tn*BVANK1 was tested on *A. pisum* 3rd instar nymphs which were fed with four different concentrations of the recombinant *Tn*BVANK1 protein (7.5, 15, 30 and 60 µg/ml) in the artificial diet formulated by Febvay et al. (1988), offered with the feeding apparatus described by Powell et al., (1993). Diet alone or supplemented with 60 µg/ml of the supernatant of non-transformed *P. pastoris* strain X-33 (produced after large scale fermentation) were used as controls. Three replicates of 10 individuals were performed for each treatment (30 aphids/treatment). The sachets containing the experimental diet were replaced every two days. Aphid mortality was daily monitored until the last death occurred at one of the tested concentrations.

3.2.3 Histological and ultrastructural analysis

To study the structural alterations induced by *Tn*BVANK1, two groups of 10 *A. pisum* 3rd instar nymphs were placed in feeding apparatus, as previously described in section 1.2.2. One group was fed with artificial diet only (control), while the other group was exposed to 100 µg/ml of recombinant *Tn*BVANK1.This concentration was chosen because in preliminary tests it led to a mortality of about 80% after 24 h. The impact of *Tn*BVANK1 on the midgut tissues of *A. pisum* was examined 6 and 12 hours after exposure to the protein, with each experiment repeated twice.

3.2.4 Optical and Transmission Electron Microscopy

The experimental 3rd instar nymphs, obtained as described above, were anesthetized on ice for 10 minutes and fixed with 2% glutaraldehyde and 0,1% Triton-X in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h and then washed three times (5 min each) with the same buffer. Specimens were postfixed with 1% osmium tetroxide in 0,1 M cacodylate buffer for 2 h at room temperature and, after standard ethanol dehydration, they were embedded in Epon-Araldite 812 mixture (Sigma-Aldrich, Milan, Italy). Sections were obtained with Reichert Ultracut S ultratome (Leica, Wien, Austria). Semithin sections (0.75-µm-thick) were stained with crystal violet and basic fuchsin, analyzed with Nikon Eclipse Ni light microscope (Nikon, Tokyo, Japan), and images were recorded with DS-5 M-L1 digital camera system (Nikon). Ultrathin sections (70-nm-thick) were collected on copper grids, stained with uranyl acetate and lead citrate, and analyzed with JEOL-1010 TEM (Jeol, Tokyo, Japan) equipped with Morada digital camera (Olympus, Tokyo, Japan) - Centro Grandi Attrezzature, University of Insubria.

3.2.5 Immunohistochemistry

Aphids were fixed with 4% paraformaldehyde and 0,1% Triton-X in phosphate buffer saline (PBS; 138 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4) for 3 h, and then washed three times (5 min each) with the same buffer. Samples were dehydrated in an increasing scale of ethanol and embedded in paraffin. Sections (7-µm-thick) were obtained with Jung Multicut 2045 microtome (Leica). Antigen retrieval was performed by treating sections with 10 mM sodium citrate buffer (pH 6.0) for 13 min in a microwave oven. Samples were preincubated with 2% bovine serum albumin (BSA) and 0.01% Tween-20 in PBS for 30 min, and then incubated with an anti-His•Tag mouse monoclonal antibody (diluted 1:100 in 2% BSA) (Novagen) to detect ANK, for 1 h at room temperature. After three washes (5 min each) in PBS, slides were incubated with an anti-mouse Cy3-conjugated secondary antibody (dilution 1:100 in 2% BSA) (Jackson ImmunoResearch Laboratories, West Grove, USA) for 45 min and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; 0.1 mg/mL in PBS) for 3 min. Samples were then mounted with Citifluor (Citifluor Ltd, London, UK) and analyzed with Eclipse Ni-U microscope (Nikon) equipped with DS-SM-L1 digital camera (Nikon). Images were superimposed with Adobe

Photoshop (Adobe Systems, San Jose, CA, USA). Negative controls were performed by omitting the primary antibody.

3.3 Statistical Analysis

A. pisum survival curves exposed to different dosage of recombinant *Tn*BVANK1 were compared by using Kaplan–Meier and Log-rank analysis, while the 10, 50 and 90% lethal concentration (LC_{10}, LC_{50} and LC_{90}) values at day 3, 4, and 5 were assessed by Probit analysis (Finney, 1971). All data were analyzed using Prism (GraphPad Software Inc. version 6.0b, San Diego, CA, United States).

3.4 Results

3.4.1 Effects of recombinant *Tn*BVANK1 on *A. pisum* survival

*Tn*BVANK1 oral toxicity was examined on *A. pisum* nymphs fed with the recombinant protein dissolved in the artificial diet at a dose ranging from 7.5 to 60 µg/mL. Results showed significantly lower survival rates for aphids alimented on *Tn*BVANK1-enriched diets compared to controls, as determined by LogRank test (X^2 = 103.6; df = 5; P<0.0001) (Figure 3.2). Recombinant TnBVANK1 resulted in 100% mortality after 6 days on the highest dose (60 µg/mL). However, both the concentration of 7.5 µg/mL and 15 µg/mL did not differ from controls (Figure 3.2). The *Tn*BVANK1 dosage causing the death of 10%, 50%, and 90% of tested aphids is reported in Table 3.1.

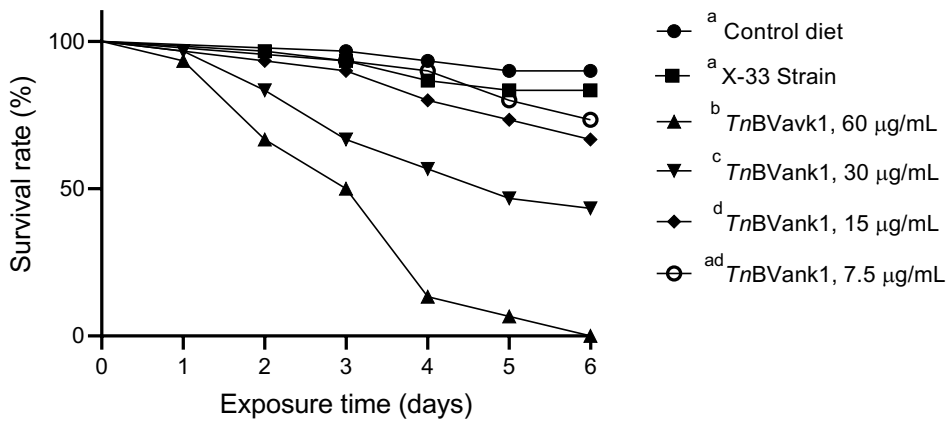

Figure 3.2 Survival curves of *Acyrthosiphon pisum* exposed to different doses of TnBVANK1. Significant differences (P<0.05) of tested diets are denoted with different letters.

Concentration	Day	LC_{10}[a]	LC_{50}[a]	LC_{90}[a]
µg/mL	3	14.78 (18.72 to 28.76)	33.34 (27.32 to 40.69)	44.87 (33.56 to 53.29)
	4	8.746 (7.145 to 10.79)	18.98 (17.15 to 21.00)	41.19 (32.21 to 50.61)
	5	4.661 (3.64 to 6.015)	13.04 (11.73 to 14.50)	36.50 (28.86 to 46.08))

Table 3.1 LC_{10}, LC_{50}, and LC_{90} calculated at the day 3, 4, and 5 for *Acyrthosiphon pisum* fed on artificial diet containing *Tn*BVANK1 recombinant protein. [a] Ninety−five percent lower and upper fiducial limits are shown in parenthesis.

3.4.2 Optical and TEM analysis of *A. pisum* midgut

3.4.2.1 Evaluation of *Tn*BVANK1 effects on the stomach.

The effects of *Tn*BVANK1 on the midgut were evaluated by means of optical and TEM analysis. In control aphids, fed on standard diet, the epithelium of the stomach was formed by lobed and pyramidal digestive cells that protruded into the lumen and showed large nuclei and few vacuoles in the cytoplasm. (Fig. 3.3 A, B). Six hours after *Tn*BVANK1 ingestion (Fig. 3.3 C), the cell structure appeared partially altered, with enlarged vacuoles in the cytoplasm. A more significant morphological change of the epithelial cells was observed after 12 hours (Fig. 3.3 D). In particular, the epithelium lost its typical organization and cells appeared more flattened, with reduced cytoplasm and oval nuclei.

TEM analysis confirmed ultrastructural changes in the gut cells 12 hours after *Tn*BVANK1 ingestion (Fig. 3.3 F). While in control insects the epithelial cells showed a complex network of lamellae, which are protrusions of the apical membrane similar to microvilli, associated with membranous masses forming the modified perimicrovillar membranes (MPM) (Fig. 3.3 E) (Cristofoletti et al., 2003), after *Tn*BVANK1 administration both lamellae and MPM were not clearly visible, and the cells appeared thinner (Fig. 3.3 F).

3.4.2.2 Evaluation of *Tn*BVANK1 effects on the proximal tubular intestine

The ingestion of *Tn*BVANK1 induced severe effects on the proximal region of the tubular intestine. The epithelium of this gut region was formed by triangular cells that abundantly protruded into the lumen, which resulted significantly reduced and star shaped (Fig 3.4 A, B). Epithelial cells contained small cytoplasmic vacuoles and ovoid nuclei.

Six hours after *Tn*BVANK1 administration (Fig. 3.4 C), cell morphology appeared partially modified. The dimension of vacuoles increased, and the intestinal lumen enlarged. A significant effect was visible after 12 hours (Fig. 3.4 D), when the intestinal epithelium resulted extensively damaged, and cells were no longer visible.
TEM analysis confirmed the consistent alteration of the intestinal epithelium. In fact, while in proximal intestine of controls the cells appeared intact with well-organized apical lamellae (Fig. 3.4 E), the ingestion of *Tn*BVANK1 induced a complete alteration of the epithelium: cells were completely destroyed and only few cytoplasmic debris were visible (Fig. 3.4 F).

3.4.2.3 Evaluation of *Tn*BVANK1 effects on the distal tubular intestine

Deleterious effects of *Tn*BVANK1 on the distal region of the tubular intestine were less marked that in the proximal part. Compared to the proximal region, the epithelium of the distal tract was composed by a greater number of cells that possessed bigger nuclei and a more vacuolated cytoplasm (Fig. 3.5 A, B). Six hours following *Tn*BVANK1 ingestion, the morphology of the epithelium resulted slightly modified (Fig. 3.5 C), showing an increase in the number of cytoplasmic vacuoles. The dimensional growth of the vacuoles persisted up to 12 hours (Fig. 3.5 D), when they fused together. Moreover, the apical region of the epithelium lost its morphology and the lamellae appeared damaged and disorganized (Fig. 3.5 D), as confirmed by TEM (Fig. 3.4 F, H). This microvilli-like network in the apical surface of control epithelium (Fig. 3.5 E, G) was less developed than in the proximal region, but well visible.

3.4.2.4 Immunostaining analysis

The localization of *Tn*BVANK1 protein was assessed in the tubular intestine of control (Fig. 3.6 A, B) and aphids which ingested the recombinant protein (Fig. 3.6 C, D) by means of immunofluorescence. No signal was detected at 6 (Fig. 3.6 A) and 12 hours (Fig. 3.6 B) in gut samples of aphids maintained on standard diet. Conversely, 6 hours after *Tn*BVANK1 ingestion (Fig. 3.6 C), a strong positivity was visible both in the apical and in the basal region of epithelial cells. Twelve hours after *Tn*BVANK1 administration (Fig. 3.6 D), since the apical region of the cells was completely damaged, the signal was mainly located in their basal region. No signal was detected in negative control experiments (Fig. 3.6 E) No signal was detected on other organs localized in the haemocoel.

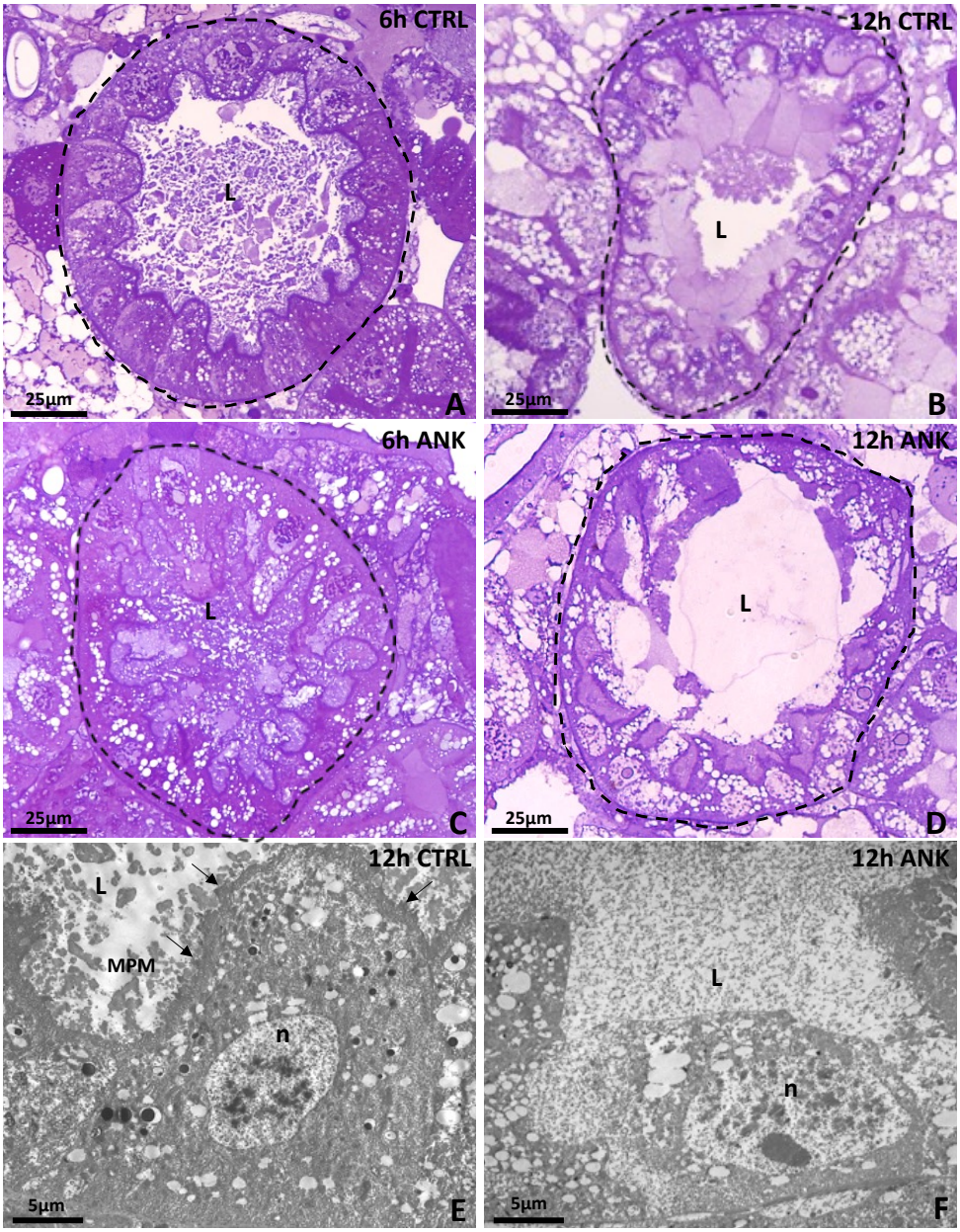

Fig. 3.3 *Tn*BVANK1 effects on the *Acyrthosiphon pisum* stomach. Optical and TEM analysis. The epithelium of this district is formed by pyramidal cells that show round nuclei (n) and few small vacuoles in the cytoplasm. In the apical region, the network of lamellae (arrows) associated to modified perimicrovillar membranes (MPM), is visible (E). After *Tn*BVANK1 ingestion (C-D,F), cell morphology results altered. The number of cytoplasmic vacuoles increases, both in number and size, and cells appear more flattened. The dashed line indicates the cross-sectioned stomach. L: lumen.

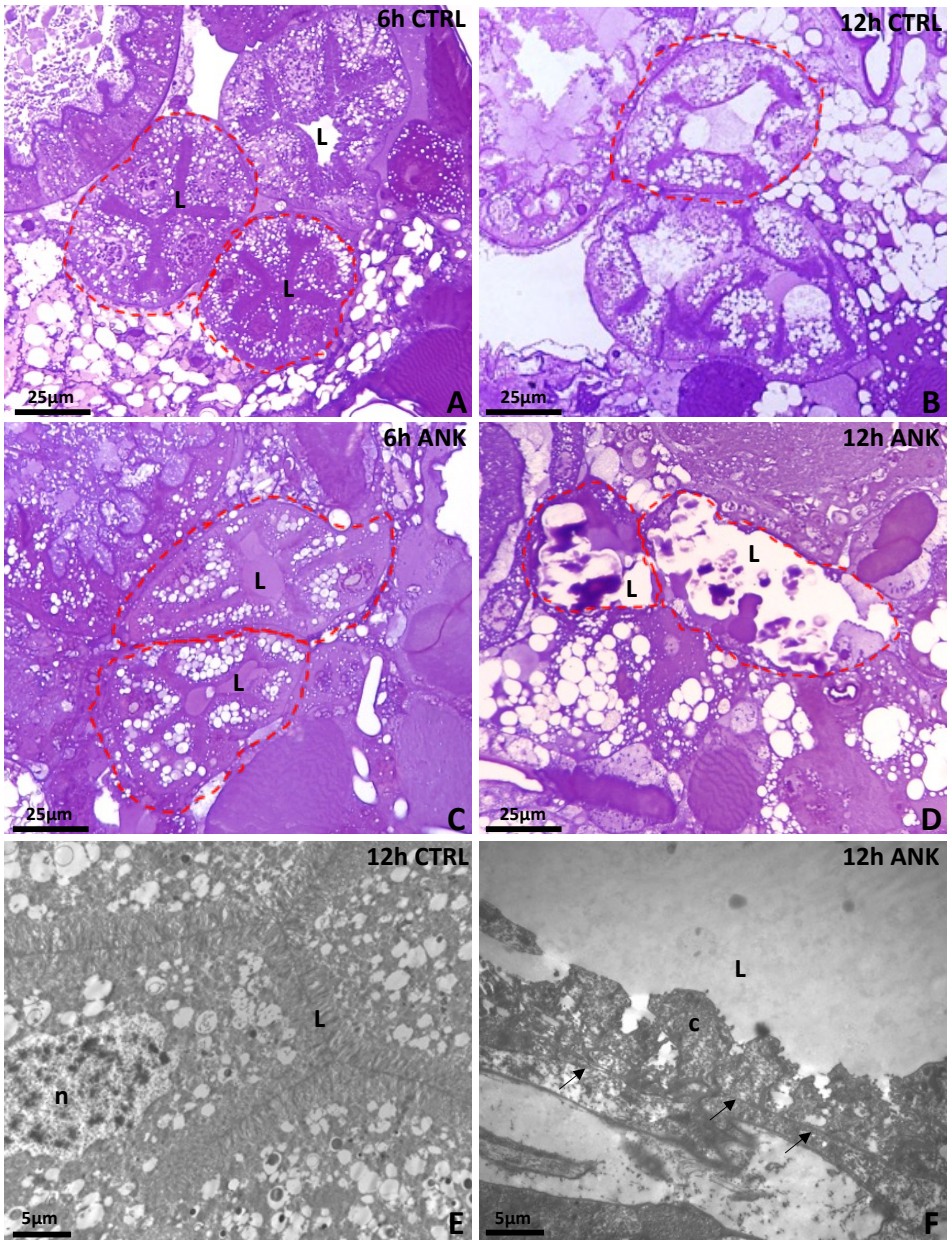

Fig. 3.4. *Tn*BVANK1 effects on the proximal tubular intestine. Optical and TEM analysis. The epithelium of the proximal tubular intestine is formed by triangular cells, protruding into a reduced and star-shaped lumen. The complex network of lamellae in this district is more compact and cells are close to each other (E). After ANK administration (C-D,F), a significant deleterious effect on this gut district is detectable especially after 12 hours (D,F), when epithelial cells result considerably damaged. Cell debris are associated to the basal lamina (arrows). The dashed line indicates the cross-sectioned proximal tubular intestine. n: nuclei. L: lumen.

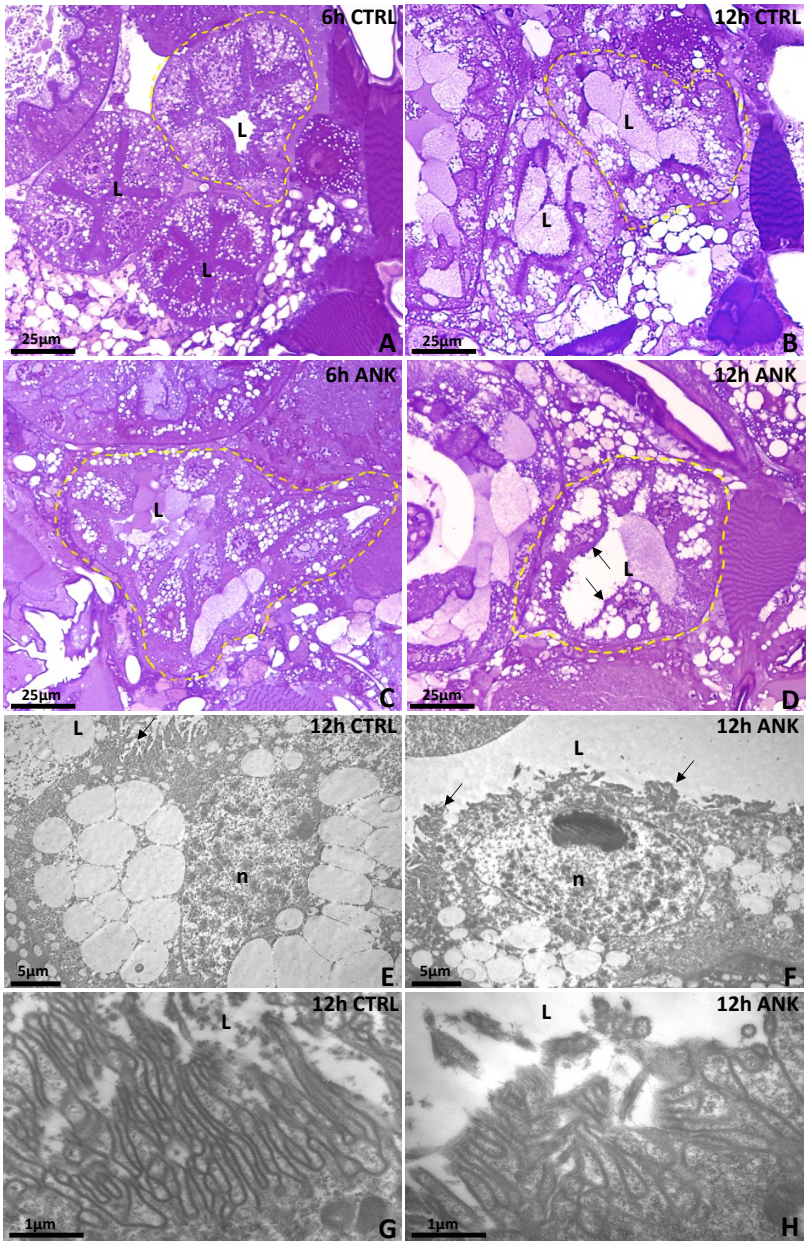

Fig. 3.5. Evaluation of *Tn*BVANK1 effects on the distal tubular intestine. Optical and TEM analysis. The epithelium of the distal tubular intestine is formed by enlarged cells and lumen, with more developed vacuoles compared to the proximal region. In this district, the complex network of lamellae (arrows) is less developed than in the proximal region, but well-visible in control samples (E,G). After *Tn*BVANK1 ingestion (C-D,F,H), the apical region loses its typical organization and lamellae appear damaged (F,H). The dashed line indicates the cross-sectioned distal tubular intestine. n: nuclei. L: lumen.

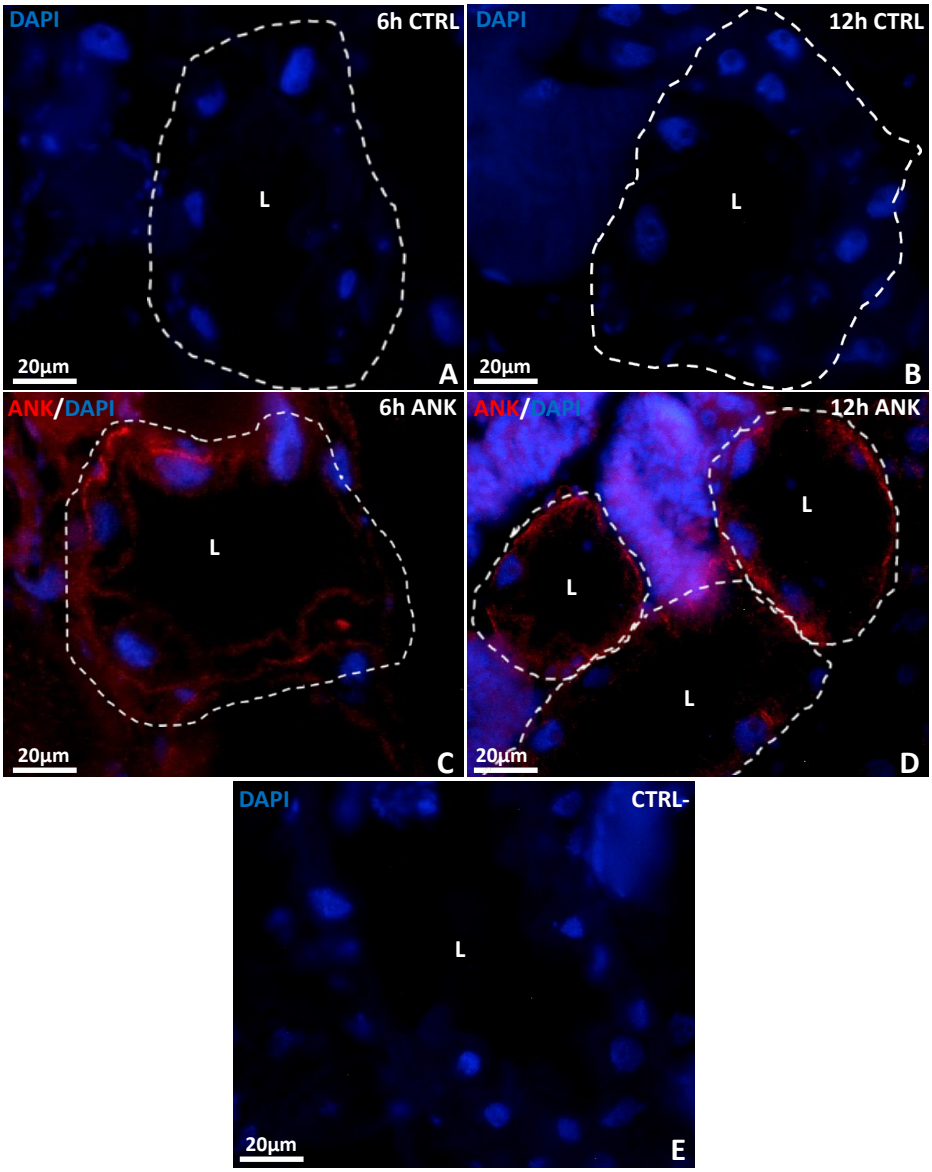

Fig. 3.6. Immunofluorescence analysis of *Tn*BVANK1. No signal is visible in control samples at 6 (A) and 12 hours (B). A strong signal, both in apical and basal region of epithelial cells, is detectable in aphids 6 hours after *Tn*BVANK1 ingestion (C). After 12 hours (D), the fluorescent signal is mainly localized in the basal region of the epithelium, due to the disruption of cell morphology. No signal is detected in negative control experiments (E). The dashed line indicates the longitudinally sectioned tubular intestine. L: lumen.

3.5 Discussion

Aphids are agricultural pests of remarkable economic importance, responsible for large yield losses in many crops (Simon and Peccoud, 2018). The strong need to reduce synthetic pesticide use in agriculture, due to their negative impact on human health and the environment, requires the development of alternative control tools, including those biotechnology-based which are particularly limited in the case of aphids (Will and Vilcinskas, 2013).

The molecular biodiversity of parasitic wasps provides an interesting opportunity to develop alternative pest management strategies, based on the use of a new category of natural bioinsecticides, which have been only limitedly exploited (Beckage and Gelman, 2004; Pennacchio et al., 2012). Regulation factors released by parasitic wasp at oviposition, such as PDV-containing calyx fluid, may be a source of molecules that, once identified and characterized, can be useful in the design of novel PDV-based biopesticides (Beckage and Gelman, 2004; Pennacchio and Strand, 2006; Pennacchio et al., 2012).

In this part of the thesis work, we studied the impact of a virulence factor belonging to the ANK protein family of the *T. nigriceps*-associated bracovirus (Falabella et al., 2007) on the pea aphid, *A. pisum* The ANK gene family is commonly widespread in both bracoviruses and ichnoviruses (Strand, 2012) and plays a key-role in disrupting the host's immune response and hormonal balance, by interacting with various cellular targets (Thoetkiattikul et al., 2005; Falabella et al., 2007; Bitra et al., 2012; Valzania et al., 2014). Specifically, we focused on *Tn*BVANK1, a virulence factor involved in both immunosuppression and disruption of ecdysone biosynthesis, when the viral-encoding gene is expressed in the tissues of parasitized hosts (Pennacchio and Strand, 2006; Valzania et al., 2014).

The oral toxicity of *Tn*BVANK1 to *S. littoralis* larvae is due to its sticking on the surface of the absorbing gut epithelium (Di Lelio et al., 2014). Here we report that *Tn*BVANK1 is orally toxic to *A. pisum* in a dose-dependent manner. Indeed, the feeding bioassays show a transition from no significant effect on survival for the lowest dose used, to 100% mortality after 6 days in aphids fed with the highest experimental dose.

Using optical microscopy and TEM analysis, the effect and localization of *Tn*BVANK1 in the midgut of aphids fed with the recombinant protein was assessed. The digestive tract of *A. pisum* includes an enlarged anterior chamber, commonly referred to as the stomach, followed by a

thinner, elongated region known as the tubular intestine, which is further divided into a proximal and distal section (Ponsen, 1991; Cristofoletti et al., 2003). We found that the ingestion of *Tn*BVANK1 alters the structure of the proximal region of the tubular intestine, where the epithelium appeared extensively damaged, with cells lacking structural integrity. These findings are in line with earlier research showing that various toxins target the proximal and distal parts of the tubular intestine of *A. pisum* (Chougule et al., 2013; Rausch et al., 2016). These changes were further supported by TEM analysis, which revealed that the cells were degraded, with only a few cytoplasmic debris visible. The deleterious effects of recombinant *Tn*BVANK1 in the distal tubular intestine were less pronounced than in the proximal region. When compared to controls, the distal tract epithelium showed a slight modification, with an increase in the number of cytoplasmic vacuoles and the apical region losing its natural morphology. *Tn*BVANK1 was detected only in the gut lumen, bound to both apical and basal region of epithelial cells, suggesting that its ingestion is not followed by transepithelial transport across the midgut. Analogous results were previously observed in *S. littoralis* larvae, in which strong immunopositivity was detected only in the midgut of insects fed with transgenic tobacco plants expressing *Tn*BVANK1, particularly in the microvillar region of both columnar and goblet cells (Di Lelio et al., 2014). The severe damage of the gut structure is certainly part of the effects induced by TnBVANK1. In the case of lepidoptera, it seems that a coating layer lining the epithelial cells of the midgut interferes with nutrients uptake (Di Lelio et al., 2014), but if and how this is also the mechanism underlying the negative effects observed on aphids remains to be studied.

In conclusion, the present work provides valuable information on the oral toxicity of *Tn*BVANK1 on *A. pisum* and reports on the major structural alterations of the aphid midgut upon its ingestion. Our findings suggest that *Tn*BVANK1 could be used as a future novel bioinsecticide to control aphids. It would be worth to assess if the observed toxicity occurs also on other plant sap-sucking insects. Moreover, it will be necessary to define effective delivery strategies and assess the risk associated with its use to move forward a possible future exploitation of this interesting bioinsecticide molecule.

References

Beckage, N.E., 1998. Modulation of immune responses to parasitoids by polydnaviruses. Parasitology 116, S57–S64.

Beckage, N.E., Gelman, D.B., 2004. Wasp parasitoid disruption of host development: implications for new biologically based strategies for insect control. Annu. Rev. Entomol. 49, 299–330.

Bitra, K., Suderman, R. J., & Strand, M. R. (2012). Polydnavirus Ank proteins bind NF-κB homodimers and inhibit processing of Relish. *PLoS pathogens*, *8*(5), e1002722.

Chougule, N. P., Li, H., Liu, S., Linz, L. B., Narva, K. E., Meade, T., & Bonning, B. C. (2013). Retargeting of the Bacillus thuringiensis toxin Cyt2Aa against hemipteran insect pests. *Proceedings of the National Academy of Sciences*, *110*(21), 8465-8470.

Cristofoletti, P. T., Ribeiro, A. F., Deraison, C., Rahbé, Y., & Terra, W. R. (2003). Midgut adaptation and digestive enzyme distribution in a phloem feeding insect, the pea aphid Acyrthosiphon pisum. *Journal of Insect Physiology*, *49*(1), 11-24.

Di Lelio, I., Caccia, S., Coppola, M., Buonanno, M., Di Prisco, G., Varricchio, P., ... & Pennacchio, F. (2014). A virulence factor encoded by a polydnavirus confers tolerance to transgenic tobacco plants against lepidopteran larvae, by impairing nutrient absorption. *PLoS One*, *9*(12), e113988.

Edwards, M. G., & Gatehouse, A. M. (2007). Biotechnology in crop protection: Towards sustainable insect control. In *Novel biotechnologies for biocontrol agent enhancement and management* (pp. 1-23). Springer, Dordrecht.

Febvay, G., Delobel, B., and Rahbé, Y. (1988). Influence of the amino-acid balance on the improvement of an artificial diet for a biotype of *Acyrthosiphon pisum* (Homoptera:Aphididae). *Can. J. Zool.* 66, 2449–2453. doi: 10.1139/z88-362.

Falabella, P., Varricchio, P., Gigliotti, S., Tranfaglia, A., Pennacchio, F., & Malva, C. (2003). Toxoneuron nigriceps polydnavirus encodes a putative aspartyl protease highly expressed in parasitized host larvae. *Insect molecular biology*, *12*(1), 9-17.

Falabella, P., Caccialupi, P., Varricchio, P., Malva, C., & Pennacchio, F. (2006). Protein tyrosine phosphatases of *Toxoneuron nigriceps* bracovirus as potential disrupters of host prothoracic gland function. *Archives of Insect Biochemistry and Physiology: Published in Collaboration with the Entomological Society of America*, *61*(3), 157-169.

Falabella, P., Varricchio, P., Provost, B., Espagne, E., Ferrarese, R., Grimaldi, A., ... & Pennacchio, F. (2007). Characterization of the IκB-like gene family in polydnaviruses associated with wasps belonging to different Braconid subfamilies. *Journal of General Virology*, *88*(1), 92-104.

Gill, T. A., Fath-Goodin, A., Maiti, I. I., & Webb, B. A. (2006). Potential uses of Cys-motif and other polydnavirus genes in biotechnology. *Advances in Virus Research*, *68*, 393-426.

Hesham, A. E. L., Kaur, T., Devi, R., Kour, D., Prasad, S., Yadav, N., ... & Yadav, A. N. (2021). Current trends in microbial biotechnology for agricultural sustainability: conclusion and future challenges. In *Current trends in microbial biotechnology for sustainable agriculture* (pp. 555-572). Springer, Singapore.

Karabörklü, S., Azizoglu, U., & Azizoglu, Z. B. (2018). Recombinant entomopathogenic agents: a review of biotechnological approaches to pest insect control. *World Journal of Microbiology and Biotechnology*, *34*(1), 1-12.

Maiti, I. B., Dey, N., Pattanaik, S., Dahlman, D. L., Rana, R. L., & Webb, B. A. (2003). Antibiosis-type insect resistance in transgenic plants expressing a

teratocyte secretory protein (TSP14) gene from a hymenopteran endoparasite (Microplitis croceipes). *Plant biotechnology journal*, *1*(3), 209-219.
Pennacchio, F., & Strand, M. R. (2006). Evolution of developmental strategies in parasitic Hymenoptera. *Annu. Rev. Entomol.*, *51*, 233-258.
Pennacchio, F., Giordana, B., & Rao, R. (2012). Applications of parasitoid virus and venom research in agriculture. In *Parasitoid Viruses* (pp. 269-283). Academic Press.
Pennacchio, F., Caccia, S., & Digilio, M. C. (2014). Host regulation and nutritional exploitation by parasitic wasps. *Current Opinion in Insect Science*, *6*, 74-79.
Poirié, M., Colinet, D., & Gatti, J.-L. (2014). Insights into function and evolution of parasitoid wasp venoms. *Current Opinion in Insect Science*, *6*, 52–60. https://doi.org/10.1016/j.cois.2014.10.004.
Ponsen, M. B. (1991). Structure of the digestive system of aphids, in particular Hyalopterus and Coloradoa, and its bearing on the evolution of filterchambers in Aphidoidea. In *Structure of the digestive system of aphids, in particular Hyalopterus and Coloradoa, and its bearing on the evolution of filterchambers in the Aphidoidea* (No. 91.5, pp. 61-61). Agricultural University.
Powell, K. S., Gatehouse, A. M. R., Hilder, V. A., & Gatehouse, J. A. (1993). Antimetabolic effects of plant lectins and plant and fungal enzymes on the nymphal stages of two important rice pests, Nilaparvata lugens and Nephotettix cinciteps. *Entomologia experimentalis et applicata*, *66*(2), 119-126.
Rausch, M. A., Chougule, N. P., Deist, B. R., & Bonning, B. C. (2016). Modification of Cry4Aa toward improved toxin processing in the gut of the pea aphid, Acyrthosiphon pisum. *PLoS One*, *11*(5), e0155466.
Salvia, R., Grossi, G., Amoresano, A., Scieuzo, C., Nardiello, M., Giangrande, C., ... & Falabella, P. (2017). The multifunctional polydnavirus TnBVANK1 protein: impact on host apoptotic pathway. *Scientific reports*, *7*(1), 1-16.
Simon, J. C., & Peccoud, J. (2018). Rapid evolution of aphid pests in agricultural environments. *Current opinion in insect science*, *26*, 17-24.
Strand, M. R. (2012). Polydnavirus gene products that interact with the host immune system. In *Parasitoid viruses* (pp. 149-161). Academic Press.
Strand, M. R., & Burke, G. R. (2014). Polydnaviruses: nature's genetic engineers. *Annual Review of Virology*, *1*, 333-354.
Thoetkiattikul, H., Beck, M. H., & Strand, M. R. (2005). Inhibitor κB-like proteins from a polydnavirus inhibit NF-κB activation and suppress the insect immune response. *Proceedings of the National Academy of Sciences*, *102*(32), 11426-11431.
Valzania, L., Romani, P., Tian, L., Li, S., Cavaliere, V., Pennacchio, F., & Gargiulo, G. (2014). A polydnavirus ANK protein acts as virulence factor by disrupting the function of prothoracic gland steroidogenic cells. *PLoS One*, *9*(4), e95104.
Vinson, S. B., & Iwantsch, G. F. (1980). Host regulation by insect parasitoids. *The Quarterly Review of Biology*, *55*(2), 143-165.
Volkoff, A. N., Drezen, J. M., Cusson, M., & Webb, B. A. (2012). The organization of genes encoding ichnovirus structural proteins. In *Parasitoid Viruses* (pp. 33-45). Academic Press.
Webb B. A. *et al*. Family Polydnaviridae in *Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of* Viruses (ed. Van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L.) 253–260 (Academic Press, 2000).
Will, T., & Vilcinskas, A. (2013). Aphid-proof plants: Biotechnology-based approaches for aphid control. *Yellow Biotechnology II*, 179-203.

4 Insecticidal activity of a fusion protein containing the spider toxin SFI6

4.1 Introduction

Bioinsecticides have attracted increasing attention as environmentally friendly alternatives to synthetic agrochemicals (Kumar et al. 2021). These insecticides use insect antagonists or their derivatives, such as toxins, to effectively control pest populations (Samada et al., 2020; Sukiran et al., 2022). Among the natural antagonists of insects, arthropods, and particularly insects, offer by far the largest repertoire of control tools, both as organisms and as a source of bioinsecticide molecules (Pennacchio et al., 2012). In this context, the use of spider venom as a source of insecticidal peptide toxins has emerged as a particularly interesting field of study. Spiders, successful insect predators, evolved a variety of toxins in their venom glands to aid in prey capture (Windley et al., 2012). These toxins are classified into several chemical categories, including polyamine-like toxins that disrupt neuromuscular transmission, low molecular weight proteins or peptides that affect ion channels and receptors, and high molecular weight neurotoxins that interact with specific presynaptic receptors (Grishin et al., 1989; Kawai et al., 1991; Lipkin et al., 2002). Polypeptides, isolated from the venom of the spider *Segestria florentina* and grouped into SFI family, are of particular interest thanks to their range of biological activity (Lipkin et al., 2002). These polypeptides are responsible for the insecticidal activity of the venom and cause flaccid paralysis, implying their use as selective calcium channels blocking molecules (Lipkin et al., 2002; Fitches et al., 2004; Bende et al., 2015).

Spiders inject venom into their prey using hypodermic needle-like fangs, so there is no evolutionary pressure for spider toxins to be effective when ingested orally (Bende et al., 2015). Indeed, it has been shown that *S. florentina* toxin 1 (SFI1) requires carrier molecules to become orally active and exert its insecticidal activity (Fitches et al., 2004). The mannose-specific lectin from snowdrops, known as Galanthus nivalis agglutinin (GNA), has proven itself as a carrier molecule, enhancing the insecticidal efficacy of insect-specific toxins (Fitches et al., 2002, 2004; Nakasu et al., 2016;). By means of fusion protein technology, these carriers allow the toxin to cross the insect gut and reach the target site in a bioactive form (Fitches et al., 2004; Sukiran et al., 2022). Furthermore, safety studies on the effects of neurotoxic peptides on non-target invertebrates, such as honey bees

and parasitoids, indicate that these molecules are unlikely to have deleterious effects on beneficial arthropods (Nakasu et al., 2014, 2016).
Chapter III of the present PhD thesis is focused on a salivary gland neurotoxin from the spider *S. florentina* (SFI6) fused to bovine serum albumin (BSA) with the aim of expanding the repertoire of potential insecticidal molecules and their carriers, for the development of novel biopesticides. The SFI6 neurotoxin is known to be a highly selective antagonist of different voltage-gated calcium channels (Lipkin et al., 2002), which are the target site of action for chemical pesticides such as pyrethroids (Meijer et al., 2014). On the other hand, BSA represents a good candidate for carrying insecticidal toxins in the hemolymph of insects, as it has been shown to efficiently cross the gut epithelium of lepidopteran larvae in an undegraded form (Casartelli et al., 2005, 2008; Caccia et al., 2012).
In this study, we produced the recombinant fusion protein SFI6-BSA, consisting of SFI6 linked to the N-terminus of BSA, and demonstrated its biological activity in different insect species. Our results show that SFI6-BSA exhibits oral toxicity to agricultural pests from various insect orders, such as *Spodoptera littoralis* (Lepidoptera: Noctuidae), *Acyrthosiphon pisum* (Homoptera: Aphididae), and *Ceratitis capitata* (Diptera: Tephritidae). These promising results in terms of control efficacy are currently being complemented by extensive risk assessment studies on different non-target organisms in order to consider their future use in sustainable IPM plans.

4.2 Materials and Methods

4.2.1 Expression system

Recombinant SFI6-BSA was expressed using *Escherichia coli* and *Pichia pastoris* microbial system. *E. coli* Dh5α was sustained in LB (Lauria Bertani) stock with a low salt content [1% (w/v) Bacto-Tryptone, 0.5% (w/v) Yeast extract, and 1% (w/v) NaCl]. For effective transformation, One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific) was given.
Transformed *P. pastoris* X33 colony, selected by resistance to the antibiotic Zeocin (Thermo Fisher Scientific), was grown for 2-3 days at 30°C in a 250 ml baffled flask with 50 ml starter culture, in an orbital shaker (200 rpm).

4.2.2 Cloning of BSA in SFI6-pGAPZαA vector

The destination vector used for the expression of recombinant proteins in *P. pastoris* system is pGAPZαA.
The standard configuration of this vector is a bi-functional system enabling replication in *E. coli* and maintenance in *P. pastoris*. pGAPZαA (4979 bp) uses the GAP promoter for the constitutive expression of recombinant proteins in *P. pastoris* and generates a protein that is fused to an *N*-terminal α-factor secretion signal.
The SFI6-BSA fusion constructs (kindly donated by Professor Angharad Gatehouse, Newcastle University, UK) were first prepared by amplification of cDNA synthesized from bovine total liver RNA (Zyagen) using the primers listed in Table 4.1.

Primer	Sequence (5'→3')	Melt. temp. (T_m)
BSA *fw*	GCGGCCGCCAGGGGTGTGTTTCGTCGAGATAC	65°C
BSA *rev*	TTCTAGAAAAACAACAAGTTTTGGACCCTCCACA	64°C

Table 4.1 Primers used for BSA amplification. The restriction enzymes *NotI* (fw primers) and *XbaI* (rev primers) recognize the underlined nucleotides.

The amplified and purified fragments were cloned in pCR 2.1 (Thermo Fisher Scientific) vector according to manufacturer's instruction and restricted with *NotI* and *XbaI* as the presence of these sites allows the insertion of the amplified fragments into the SFI6-pGAPZαA vector.
The digestion reaction was performed using 1 µl of enzyme per µg of DNA and 10% of the final volume of 10x Fast Digest Buffer. After 30 minutes of incubation at 37°C, the fully digested DNA was inactivated by 5 minutes of incubation at 80°C. QIAquick Gel extraction Kit (Qiagen) was used to separate restriction fragments from the agarose gel, and the digested fragments were then ligated to SFI6-pGAPZαA (also similarly digested) in a reaction with an insert:vector ratio of 3:1.
To ensure complete ligation, reactions were incubated overnight at room temperature (RT). Furthermore, ligated constructs were used to transform One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher). To select transformants carrying the pGAPZαA plasmid, the transformation reaction was plated on LB agar plates (1% (w/v) Bacto-Tryptone, 0.5% (w/v) Yeast extract, 1% (w/v) NaCl, and 1.5% (w/v) Agar) and incubated overnight. QIAprep Spin Miniprep Kit (Qiagen)

was used to extract plasmids, and positive transformants were confirmed using colony PCR and DNA sequencing.

4.2.3 Preparation of *Pichia pastoris* competent cells and construct expression

The *Pichia* EasyComp Kit (Thermo Fisher Scientific) was used to obtain competent *P. pastoris* cells by using the procedure below. The X33 *P. pastoris* strain was streaked onto a YPD Agar plate (1% (w/v) BactoYeast extract, 2% (w/v) BactoPeptone, 2% (w/v) Dextrose, and 2% (w/v) Agar) to generate single colonies. For 2-4 days, the plate was incubated at 30°C. A single *P. pastoris* colony was inoculated in 10 ml of YPD and grown overnight in a shaking incubator at 28-30°C (250-300 rpm). The overnight culture was diluted to an OD_{600} (Optical Density) of 0.1-0.2 in 10 ml of YPD and grown in a shaking incubator at 28-30°C until the OD_{600} reached 0.6-1.0. The cells were pelleted by centrifugation at 500 x g for 5 minutes at RT, the supernatant was discarded, and the pellet was resuspended in 10 ml of the kit's Solution I.

Prepared constructs SFl6-BSA (Figure 4.1) was inserted into *P. pastoris* genome for over-expression of recombinant proteins. BamHI FastDigest (Thermo Fisher Scientific) was used to linearize the DNA construct, with 1 µl of enzyme per µg of plasmid DNA and 10% of the final volume of 10x Fast Digest Buffer.

Linear DNA can produce stable *P. pastoris* transformants through homologous recombination between the transforming DNA and homology regions within the genome (Cregg et al., 1985). Three µg of the linearized expression vector were used to transform the yeast competent cells. The DNA/cell combination was mixed after 1 ml of Solution II was added. The transformation reactions were vortexed every 15 minutes while being incubated for 3 hours at 30°C in a water bath. The cells were heat-shocked for 10 minutes in a 42°C heat-block before being divided into two microcentrifuge tubes (approximately 525 µl each) and 1 ml of YPD medium added to each tube. After incubating at 30°C for 1 hour to allow zeocin resistance expression, they were pelleted by centrifugation at 3,000 x g for 5 minutes at room temperature; after discarding the supernatant, the pellet was resuspended in Solution III.

The previous step was repeated, and the cell pellet was finally resuspended in 150 µl of Solution III. The entire transformation reaction was plated on YPD Agar plates with zeocin (100 µg/ml) and incubated at 30°C until colonies appeared on the plate (2 - 4 days). Six conspicuous colonies were randomly selected from the YPD agar

plate and cultured in YPD media for 24-36 hours. Glycerol stocks were made from these cultures and stored at -80°C until they were used to protein production.

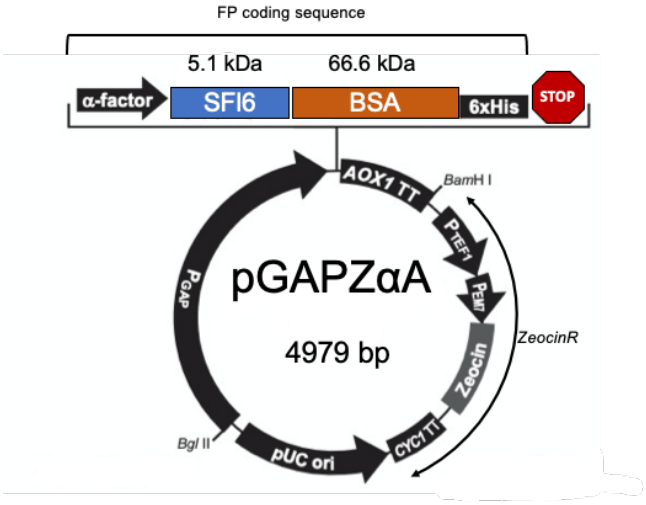

Fig. 4.1 The map of recombinant plasmid pGAPZαA. This vector uses the *GAP* promoter to constitutively express recombinant proteins in *P. pastoris*. Predicted protein size of fusion protein is approximately 72 kDa with 5.1 kDa of SFI6 and 66.6 kDa of BSA.

4.2.4 Protein expression and purification from *P. pastoris*

Selected positive colonies were cultured in YPD growth media together with the positive control expressing SFI6 toxin fused with BSA and grown in 200 ml of starter cultures in a 1L baffled flask for 2-3 days at 30°C with shaking. The starter culture was used to inoculate 900 ml of basal media (231 mM phosphoric acid, 6.83 mM calcium sulphate, 70 mM potassium sulfate, 60.4 mM magnesium sulfatex7H_2O, 73.6 mM potassium hydroxide, 435 mM glycerol, 0.03% (v/v) antifoam (Thermo Fisher)) in a bench top fermenter (New Brunswick Scientific Bioflo 110). The bench fermenter with basal media was calibrated with a pH probe and dissolved oxygen and sterilised in an autoclave at 121°C, 15 lbs pressure for 20 minutes. The sterilised fermenter vessel was then set up for measuring pH and temperature using a digital pH controller and digital temperature sensor. Cooling of the water supply was used to maintain the temperature at 30°C. Two 500 ml bottles were connected to the fermenter for the addition of acid and base, and one 1000 ml bottle for the addition of sterile 50% glycerol solution (v/v with distilled water), containing 9.6 ml PTM1 (24 mM cupric sulfatex5H_2O, 0.53 mM sodium iodide, 17.7 mM manganese sulfatexH_2O, 0.8 mM sodium molybdatex2H_2O, 0.3 mM boric acid, 4.9 µM cobalt chloride, 147 mM

zinc chloride, 243 mM ferrous sulfatex7H_2O, 0.8 mM biotin, 51 mM sulfuric acid). After 24 hours of incubation, fermentation was initiated. Sterile media supplemented with (3.92 ml/l) PTM1 salts was inoculated with 100 ml starter cultures. Cultivation was set up with input of the following parameters: 30% dissolved oxygen, pH 4.5, 30°C. A glycerol feed (4-9 ml/h) was maintained during the fermentation process. A decrease in glycerol feed and increase in the level of dissolved oxygen makes the termination of the process.
Selected proteins, contained in the supernatant, were separated from the cell pellet by centrifugation at 8,000 x g for 30 min at 4°C.

4.2.5 Nickel-chromatography purification of fusion protein

Constructs were designed with a fused *C*-terminal 6xHis tag motif so they can be purified by affinity chromatography. It is based on the interaction between Ni^{2+} ions immobilized on a matrix and the histidine side chain on the tagged protein using Ni^{2+}-NTA (nickel-nitrile-triacetic acid) affinity column. As a first step, the Ni^{2+}-NTA column is charged by washing NTA resin with 50 mM EDTA (Ethylenediaminetetraacetic acid) (containing 0.1% (w/v) SDS, pH 8) followed by distilled water to remove EDTA. The column was equilibrated with 1x lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8), and then the supernatant diluted with lysis buffer to a final concentration of 1x was loaded onto the Ni^{2+}-NTA column. Before washing the column 1x lysis buffer was loaded again. Then, the loaded column was washed with 50 ml of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, pH 8). Finally, bound proteins were eluted with 15 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, pH 8).
Each single fraction generated from the load, wash and elution steps was collected and analysed using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). After the end of protein purification, the column was stripped and rinsed following the manufacturer's recommendations. The soluble fractions of the protein were dialysed against distilled water at 4°C using dialysis tubing with a molecular weight cut off of 12-14 kDa. Dialysed proteins were transferred to a round bottom flask and then it was frozen in liquid nitrogen and freeze dried. The obtained samples were checked by Western-Blot analysis.

4.2.6 Insect rearing

Spodoptera littoralis is permanently lab-reared at the Department of Agricultural Sciences and derives from a population collected on flower crops in Agro-Pontino (Latina, Italy). Cotton leafworm larvae were reared on an artificial diet (41.4 g/l wheat germ, 59.2 g/l brewer's yeast, 165 g/l corn meal, 5.9 g/l ascorbic acid, 1.53 g/l benzoic acid, 1.8 g/l methyl-4-hydroxybenzoate, and 29.6 g/l agar), as previously described in Di Lelio et al., 2014, at 25 ± 1°C, 70 ± 5% relative humidity, with a photoperiod of 16:8 h (L:D).
The pea aphid *A. pisum* was reared on broad bean (*Vicia faba*) plants in the laboratory without any exposure to chemical or biological pesticides. The *A. pisum* line originates from alfalfa grasslands collected in Eboli (Salerno province), Southern Italy. Aphids were reared in entomological cages (40 x 25 x 25 cm), each containing broad bean plants with 1-3 fully expanded leaves and grown in ready potting mix in plastic pots (9 cm diameter). All aphid developmental stages were maintained in a climate room at 20 ± 1°C, 75 ± 5% relative humidity, and photoperiod of 16:8 h (L:D).
Wild type *C. capitata* line was reared as described in Sasso et al. (2020), using the *Benakeion* strain obtained from the Department of Biology, University of Naples “Federico II”. Adult flies were reared in a Bugdorm (BioQuip) cage (30 x 30 x 30 cm) with free access to a dry mixture of yeast/sucrose powder (1:3) and water aside. The laid eggs were recovered through the mesh walls of the cage and placed on 30 ml of a solidified artificial diet (Sasso et al., 2020) in Petri dishes (9 cm) and after 7–10 days the newly formed puparia were reinserted in the cage to maintain a density of around 200-300 adults. All developmental stages of *C. capitata* were grown under standard laboratory conditions in a climate chamber at 25 ± 1°C, 70% ± 5% RH and photoperiod of 16:8 h light/dark.

4.2.7 Bioassays

4.2.7.1 Spodoptera littoralis

Toxicity of yeast extract containing SFI6-BSA fusion protein (freeze-dried extract re-suspended in water) towards *S. littoralis* larvae was evaluated through feeding bioassays on artificial diet, performed at the rearing condition described above. Groups of 20 newly hatched larvae (at the 1st day of the 1st instar) were isolated into a single well of a 4-wells plastic rearing trays (RT32W, Frontier Agricultural Sciences, Pitman, NJ, United States) closed by perforated plastic lids (RTCV4, Frontier Agricultural Sciences, Pitman, NJ, United States). The

experimental larvae, for 9 consecutive days, at 24 h intervals, were offered a small piece of diet with the upper surface (0.25 X 0.25 cm) uniformly overlaid with 20 µl of a solution containing SFl6-BSA fusion protein in water (w/v) at 4 different concentrations obtained by serial dilution (2.8 to 22.4 µg/cm^2). The pieces of diet were daily replaced, and the experiment was repeated four time, for a total of 80 larvae. As controls, pieces of diet (1 cm^2) with the upper surface uniformly overlaid with 50 µl of denatured SFl6-BSA solution (obtained boiling the solution 10 minutes at 100°C) at the higher concentration or with water alone were offered to larva. Larval mortality was daily recorded.

4.2.7.2 *Acyrthosiphon pisum*

The effect of recombinant SFL6-BSA on *A. pisum* survival was assessed using an artificial feeding bioassay in which 2nd instar nymphs were exposed to serial concentrations of the fusion protein. The feeding apparatus was designated as in Powell et al., (1993), which included a liquid artificial diet specific for the pea aphid (Figure 4.2), prepared according to Febvay et al., (1988). To achieve synchronized individuals, 20 reproductive females were removed from the laboratory stock colony and placed on artificial diet for 12 hours. The offspring produced were used in bioassays when they reached the second instar. *A. pisum* nymphs were exposed to four serial concentrations of SFL6-BSA in artificial diet (20, 10, 5, 2,5 µg/ml). Diets alone or supplemented with 2 µg/ml of BSA and 2 µg/ml of denatured SFL6-BSA (heated to 100°C for ten minutes) were employed as comparable controls. Four replicates of 10 individuals were performed for each treatment and all experimental diets were replaced every two days. Aphid mortality was recorded daily until the last death occurred at any of the concentrations investigated. At the end of the experiments, survival curves and the approximate concentrations that killed 10, 50 and 90% of tested aphids were calculated using the mortality data. The tests were carried out in a separate environmental chamber under the same trial conditions as previously described.

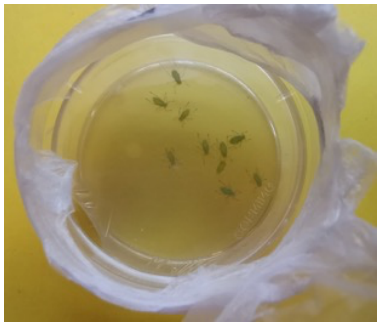

Fig. 4.2 Aphids fed on artificial diet presented in Parafilm sachets. The feeding apparatus was designated as in Powell et al., (1993), which included a liquid artificial diet specific for the pea aphid (Febvay et al., 1988).

4.2.7.3 *Ceratitis capitata*

The SFL6-BSA lethal effect on *C. capitata* larvae was evaluated in a semisolid artificial diet based on sterile homogenized fruit, prepared by mixing 20% selected yeast powder and 0.2% methylparaben in ethanol (w/v). The yeast was previously treated at 100°C for 10 minutes to inactivate any proteolytic enzymes in the powder formulation. The recombinant SFL6-BSA was added to the diet in 4 serial concentrations (32.5, 75, 112.5, and 150 μg/g). Three controls were included in experiments, consisting artificial diet garnished with denatured SFl6-BSA or BSA alone (both 150 μg/g) and non-garnished fusion protein. Each experimental diet was presented to ten neonate medfly larvae, in petri dishes (35 mm×10 mm) (Figure 4.3) and kept under controlled temperature of 20 ± 1°C and 60-70% ± 5% RH in a climate chamber. The bioassay was realized in triplicate (30 individuals/treatment) and larval mortality was assessed daily.

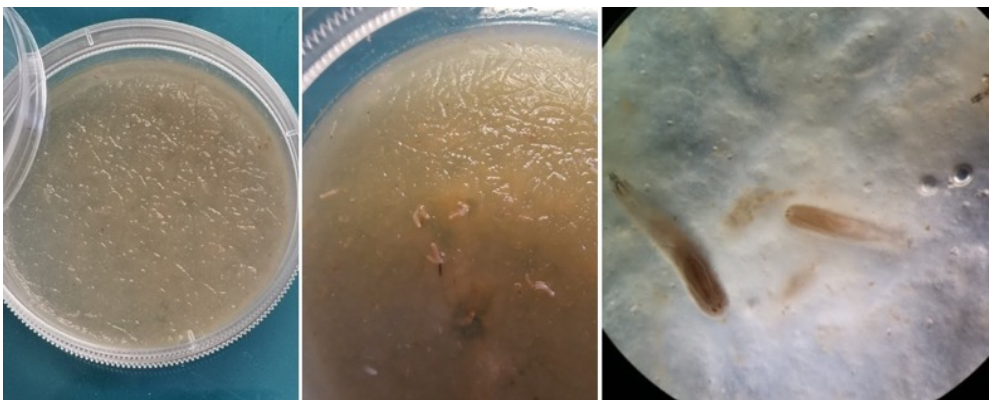

Fig. 4.3. *Ceratitis capitata* experimental larvae maintained on petri dishes containing semisolid artificial diet.

4.3 Statistical Analysis

Survival curves of *S. littoralis, A. pisum*, and *C. capitata* were compared by using Kaplan–Meier and Log-rank analysis, while the 10, 50 and 90% lethal concentration (LC_{10}, LC_{50} and LC_{90}) values at day 4, 5, and 6 were assessed by Probit analysis (Finney, 1971). All data were analyzed using Prism (GraphPad Software Inc. version 6.0b, San Diego, CA, United States).

4.4 Results

4.4.1 Purification of recombinant SFI6-BSA

The SFI6-BSA fusion protein was purified through a two-step method that involved hydrophobic interaction chromatography and affinity chromatography on a Nickel column. The purified protein was obtained through TCA precipitation following separation from the filtered culture supernatant through hydrophobic interaction chromatography. Then a WB with anti-His antibodies was performed to analyze the purified protein. The analysis revealed the presence of a major protein with molecular weight of about 70 kDa, which resulted similar to the both positive control (Figure 4.4). However, several lower molecular weight bands representing degraded protein with C-terminal sequences containing His residues were found. The 6XHis tag at the C-terminal end of the fusion protein allowed further purification using affinity chromatography on a Nickel column. Finally, WB analysis highlighted the purest fraction containing the SFI6-BSA with only two main bands corresponding to the positive control. To increase yield, the expression cassette was re-engineered to express multiple copies of the gene, allowing for a higher production of the SFI6-BSA, which was then purified using the same methods.

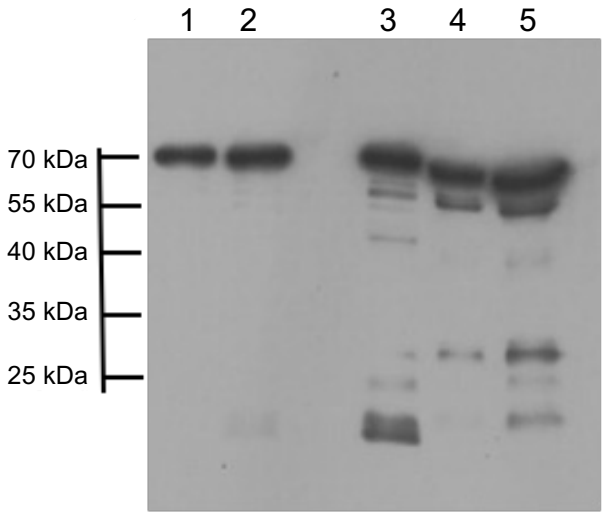

Fig. 1 Recombinant fusion protein SFI6-BSA from TCA precipitation of supernatant from fermentation by Western Blot. Blot was probed with anti-His antibody. Lanes 1, 2: positive control (10 μl of fusion protein from flask culture); lane 3: TCA precipitated protein from starter culture (10 μl); lanes 4, 5: TCA precipitated protein from fermentation (10 and 20 μl, respectively).

4.4.2 Oral toxicity of SFI6-BSA to *S. littoralis*

The insecticide activity upon ingestion of SFI6-BSA by *S. littoralis* larvae was assessed on artificial diet, using dosages ranging from 2.8 to 22.4 μg/cm^2.

The results showed that *S. littoralis* larvae had a higher mortality in treatments with SFI6-BSA, starting from the third day after the onset of the bioassay. Survival curves indicated a clear dose/response effect and resulted significantly different among them (LogRank test; $X^2 = 528.8$; df = 6; $P<0.0001$) (Fig. 4.5). Multiple comparisons revealed statistical differences ($P<0.05$) among all treatments in which SFI6-BSA was administered. The controls, consisting of BSA alone and artificial diet with water or denatured SFI6-BSA, did not significantly affect the survival, showing a clear insecticide activity of the tested fusion protein, which, 10 days after the onset of the bioassay, resulted in 100% and 71.5 % mortality at the highest (22.4 μg/cm^2) and lowest dose (2.8 μg/cm^2), respectively (Fig. 4.5). The amount of fusion protein which caused the death of 10, 50, and 90% of tested *S. littoralis* larvae is reported in Table 4.2.

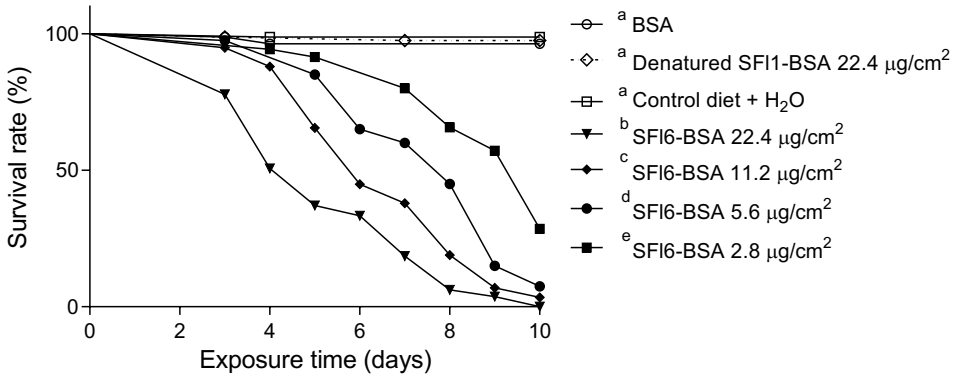

Figure 4.5. Survival curves of *Spodoptera littoralis* larvae exposed at different SFI6-BSA doses. Significant differences (P<0.05) of tested diets are indicated by different letters.

4.4.3 Oral toxicity of SFI6-BSA to *A. pisum*

A feeding bioassay was carried out on liquid artificial diet to test the insecticide activity of SFI6-BSA on *A. pisum*, showing a highly significant effect of the experimental treatment (X^2 = 193.3; df = 5; P<0.0001) (Fig. 4.6). Pairwise multiple comparisons (P<0.05) showed that the survival curves of pea aphid cohorts fed on SFI6-BSA enriched diets at different doses were all significantly different among them and compared to controls, which showed a very similar and negligible mortality rate (Fig. 4.6). SFI6-BSA resulted in 100% mortality 8 days after the onset of the bioassay, when aphids were fed the highest dose of 20 µg/mL of fusion protein, while the lowest dose of SFI6-BSA (0.5 µg/mL) determined a mortality of 19.5%. In Table 4.2 are reported the lethal concentrations of SFI6-BSA, calculated at day 4, 5, and 6.

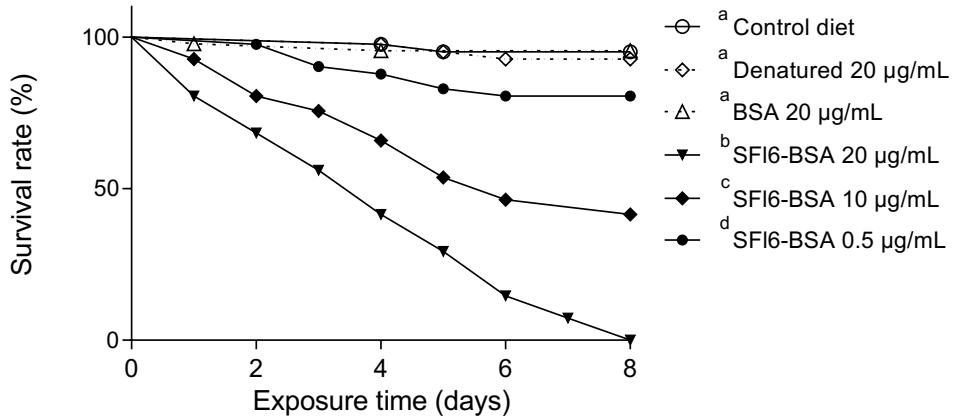

Figure 4.6. Survival curves of *Acyrthosiphon pisum* exposed at different SFI6-BSA doses. Significant differences (P<0.05) among tested diets are denoted with different letters.

4.4.4 Oral toxicity of SFI6-BSA to *C. capitata*

In Figure 4.7 is reported the overall survival of *C. capitata* larvae in the assays where different dosages of SFI6-BSA fusion protein was incorporated (32.5 to 150 µg/g) into the semisolid artificial diet. Kaplan–Meier log-rank survival analysis showed significant differences among the survival curves (X^2 = 171.7; df = 6; P<0.0001). Larvae treated with the highest dose (150 µg/g) of SFI6-BSA showed a 100% mortality on the 7th day after the onset of the bioassay. Medfly larvae fed on control diet showed low mortality over the 7 days of the bioassay, with over 92% of larvae still alive in control diet. Larvae fed on the diet enriched with BSA or with denatured SFI6-BSA showed similar survival rates. The lowest dose of fusion protein (32.5 µg/g) resulted in a larval mortality of 26.7% on day 7th. Table 4.2 shows the amount of SFI6-BSA that killed 10, 50, and 90% of the tested *C. capitata* larvae.

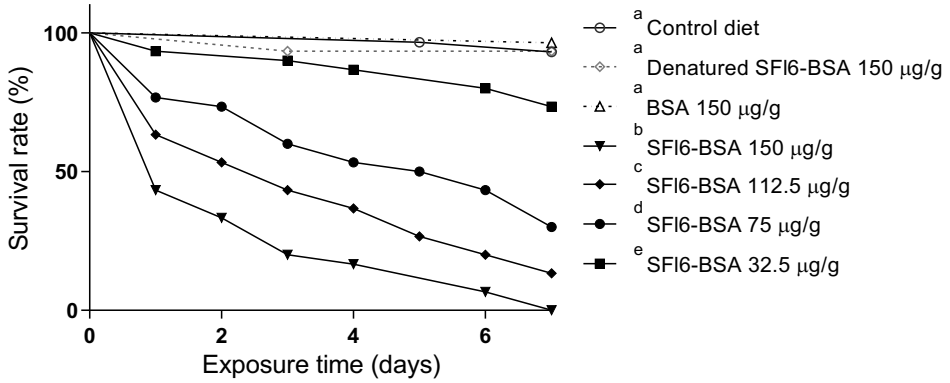

Figure 4.7. Survival curves of *Ceratitis capitata larvae* exposed at different SFI6-BSA doses. Significant differences (P<0.05) of tested diets are denoted with different letters.

Insects	Concentration	Day	LC_{10}[a]	LC_{50}[a]	LC_{90}[a]
S. littoralis	µg/cm^2	4	8.169 (6.931 to 9.367)	12.08 (11.40 to 12.81)	17.87 (14.69-21.78)
		5	5.985 (5.319 to 6.764)	10.03 (9.529-10.49)	16.81 (15.26-18.57)
		6	3.801 (3.419-4.240)	7.73 (7.325−8.148)	15.70 (12.81−17.11)
A. pisum	µg/mL	4	5.35 (4.150 to 6.899)	10.05 8.976 to 11.26	20.62 17.87 to 23.29
		5	4.349 (3.083 to 6.181)	9.47 (8.012 to 11.19)	19.42 (14.56 to 21.34)
		6	3.815 (2.862 to 5.130)	8.825 (7.730 to 10.08)	18.89 (14.63 to 20.03)
C. capitata	µg/g	4	36.60 (27.72-49.47)	71.94 (63.52-81.49)	141.4 (115.1-161.31)
		5	30.21 (21.83-43.56)	61.89 (52.12-73.49)	126.8 (98.50-137.13)
		6	23.71 (18.24-31.80)	52.60 (45.67-60.58)	116.7 (92.97-145.5)

Table 4.2. LC_{10}, LC_{50}, and LC_{90} calculated at the day 4, 5, and 6 for *Spodoptera littoralis*, *Acyrthosiphon pisum*, and *Ceratitis capitata* fed on artificial diet containing SFI6-BSA fusion protein. [a] Ninety−five percent lower and upper fiducial limits are shown in parenthesis.

4.5 Discussion

Biotechnology offers the opportunity to develop increasingly innovative biopesticides which are crucial in the implementation of sustainable control methods in agriculture (Mateos Fernández et al.,

2022), as they allow to harness the potential of natural predators and parasitoids to regulate pest populations and maintain the sustainability of crops (Pennacchio et al., 2012; Alemu, 2020). Arthropods, including predators like spiders and scorpions, act as effective natural antagonists of insects due to their evolved range of molecular strategies, including venom, which they use to kill or manipulate the physiological processes of their victims (Pennacchio and Strand, 2006; Pennacchio et al., 2012). Research on the use of spider venom as a source of neurotoxins is receiving more attention as it was seen as a valuable resource for developing new biopesticides (Windley et al., 2012; Bende et al., 2015). However, crop protection typically involves oral application of insecticidal molecules whilst venom components are not orally active since they are evolutionary meant to be directly injected into the body of their prey (Pennacchio et al., 2012; Sukiran et al., 2022). After being ingested by target insects, toxic proteins must escape the proteolytic processes occurring in the gut lumen and be transported in a bioactive form into the body cavity (Fitches et al., 2004; Pennacchio et al., 2012). Once released into the haemolymph in an undegraded form, these peptides can reach their target tissues and exert their functions (Fitches et al., 2004; Pennacchio et al., 2012, Yang et al., 2014).

In this PhD work, we focused on the development of an efficient delivery strategy for SFI6, a neurotoxin derived from the venom of the tube web spider *S. florentina*, which selectively blocks insect calcium channels (Lipkin et al., 2002). The purpose was to produce a fusion protein comprising SFI6 linked to the N-terminus of the carrier protein BSA using *P. pastoris* as expression host, and subsequently to conduct a comparative assessment of insecticidal efficacy towards different species of phytophagous insects via feeding bioassays. The recombinant SFI6-BSA fusion protein was successfully produced in yeast cells using the pGAPZαA expression vector and purified using nickel affinity chromatography. The purified SFI6-BSA was found to be immunoreactive with anti-His antibodies, indicating successful expression and purification of the protein.

The insecticidal activity of SFI6-BSA was evaluated in oral toxicity trials against *S. littoralis* and *C. capitata* larvae, and nymphs of *A. pisum*, in dose response studies as described in Nakasu et al., 2014b. We reported that SFI6-BSA has insecticidal effect on all three species, starting from the 1st to 3rd day after the onset of the bioassay and showing a clear dose-response relationship, observed in all experimental insects. The highest mortality rates were achieved at the highest doses of SFI6-BSA, with 100% mortality observed in *S.*

littoralis and *A. pisum* after 10 and 8 days, respectively, and in *C. capitata* after 7 days. The relatively rapid insecticidal activity suggests that SFI6-BSA could effectively control these insect populations in a short time period. It is also worth noting that the lowest doses of SFI6-BSA tested in this study still resulted in a significant level of mortality. In the case of *S. littoralis*, the lowest dose tested (2.8 µg/cm^2) resulted in 71.5% mortality within 10 days. Similarly, in the case of *A. pisum*, the minimal amount of fusion protein (0.5 µg/mL) resulted in 19.5% mortality within 8 days, while in the case of *C. capitata*, the minimum dosage (32.5 µg/g) resulted in 26.7% mortality on 7th day. This implies that even small amounts of SFI6-BSA may be effective in regulating the populations of tested species.

Although several spider venom peptides have been studied for their neurotoxicity against different insect species (Fitches et al., 2012; Nakasu et al., 2014b; Pyati et al., 2014; Yang et al., 2014, 2015; Sukiran et al., 2022), the insecticidal activity of a segestritoxin (SFI) was observed in SFI1 (*S. florentina* toxin 1), when orally delivered as recombinant fusion protein (Fitches et al., 2004; Down et al., 2006). In this study we investigated for the first time the insecticidal activity of SFI6, and the reported data resulted comparable to those shown by Fitches et al. (2004), who examined the toxicity of the SFI1-GNA fusion protein towards lepidoptera larvae. This study showed that newborn larvae of the tomato moth, *Lecanobia oleracea*, fed 2.5% fusion protein enriched diet, suffered 100% mortality after 6 days. Similarly, SFI1-GNA resulted orally toxic to sap-sucking insects, as the peach-potato aphid *Myzus persicae* (Down et al., 2006). Our results showed that neurotoxic activity of SFI6-BSA fusion protein induced a mortality similar to SFI1-GNA when administered to insects belonging to the same order. This is not surprising, considering that both segestritoxins are highly conserved and have a similar mechanism of action (Lipkin et al. in 2002).

When we looked at control groups, consisting of either BSA alone or artificial diet with water or denatured SFI6-BSA, no significant effect on the survival of the insects was found. This indicated that the insecticidal activity of the fusion protein appeared specific and not induced by other factors in the artificial diet. No differences in mortality were observed between water and BSA controls, strongly supporting the hypothesis that BSA does not affect insect survival when orally ingested, as opposed to what happens when using the GNA carrier, which was found to be harmful in some cases when administered orally to insects (Rahbé et al., 1995; Down et al., 1996; Sauvion et al. 1996). Therefore, BSA proves to be a good candidate for carrying

insecticidal toxins in insect haemolymph, as previous studies have suggested (Casartelli et al., 2005, 2008).

One potential limitation of this study is the use of artificial diet as the means of administering SFI6-BSA to the pests. These methods may not accurately reflect its effectiveness in natural feeding habits and field scenarios. Further research on alternative delivery methods, such as the potential applications of nanotechnology in pesticide formulation (Kah et al., 2013), and field studies may be necessary to fully understand the potential of SFI6-BSA as an insecticide.

In conclusion, we demonstrated that the venom-derived neuropeptide SFI6 has oral toxicity against lepidopteran, hemipteran, and dipteran pests, when linked to BSA. Thus, a fusion protein-based approach may offer an opportunity to significantly enhance oral efficacy of toxins of arthropod origins. Overall, the results of this study suggest that SFI6-BSA is a promising candidate for its development as biopesticide molecule, with strong toxic effects on a wide range of relevant pest in agriculture. Further research will be necessary to fully understand the mechanisms of action and its impact on non-target organisms of this protein.

References

Alemu, M. (2020). Trend of biotechnology applications in pest management: a review. *International Journal of Applied Sciences and Biotechnology*, *8*(2), 108-131.

Bende, N. S., Dziemborowicz, S., Herzig, V., Ramanujam, V., Brown, G. W., Bosmans, F., Nicholson, G. M., King, G. F., & Mobli, M. (2015). The insecticidal spider toxin SFI1 is a knottin peptide that blocks the pore of insect voltage-gated sodium channels via a large β-hairpin loop. *The FEBS Journal*, *282*(5), 904–920. https://doi.org/10.1111/febs.13189.

Caccia S, Van Dammeb EJM, De Vos WH, Smagghe G (2012). Mechanism of entomotoxicity of the plant lectin from *Hippeastrum* hybrid (*Amaryllis*) in *Spodoptera littoralis* larvae. *Journal of Insect Physiology* 58:1177-1183.

Casartelli, M., Corti, P., Leonardi, M. G., Fiandra, L., Burlini, N., Pennacchio, F., & Giordana, B. (2005). Absorption of albumin by the midgut of a lepidopteran larva. *Journal of insect physiology*, *51*(8), 933-940.

Casartelli, M., Cermenati, G., Rodighiero, S., Pennacchio, F., & Giordana, B. (2008). A megalin-like receptor is involved in protein endocytosis in the midgut of an insect (Bombyx mori, Lepidoptera). *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *295*(4), R1290-R1300.

Di Lelio, I., Varricchio, P., Di Prisco, G., Marinelli, A., Lasco, V., Caccia, S., et al. (2014). Functional analysis of an immune gene of *Spodoptera littoralis* by RNAi. *J. Insect Physiol.* 64, 90–97. doi: 10.1016/j.jinsphys.2014.03.008

Down, R. E., Gatehouse, A. M., Hamilton, W. D., & Gatehouse, J. A. (1996). Snowdrop lectin inhibits development and decreases fecundity of the glasshouse potato aphid (Aulacorthum solani) when administered in vitro and

via transgenic plants both in laboratory and glasshouse trials. *Journal of insect physiology*, *42*(11-12), 1035-1045.

Febvay, G., Delobel, B., and Rahbé, Y. (1988). Influence of the amino-acid balance on the improvement of an artificial diet for a biotype of *Acyrthosiphon pisum* (Homoptera:Aphididae). *Can. J. Zool.* 66, 2449–2453. doi: 10.1139/z88-362.

Fitches, E., Audsley, N., Gatehouse, J. A., & Edwards, J. P. (2002). Fusion proteins containing neuropeptides as novel insect contol agents: snowdrop lectin delivers fused allatostatin to insect haemolymph following oral ingestion. *Insect Biochemistry and Molecular Biology*, *32*(12), 1653-1661.

Fitches, E., Edwards, M. G., Mee, C., Grishin, E., Gatehouse, A. M. R., Edwards, J. P., & Gatehouse, J. A. (2004). Fusion proteins containing insect-specific toxins as pest control agents: Snowdrop lectin delivers fused insecticidal spider venom toxin to insect haemolymph following oral ingestion. *Journal of Insect Physiology*, *50*(1), 61–71.

Fitches, E. C., Bell, H. A., Powell, M. E., Back, E., Sargiotti, C., Weaver, R. J., & Gatehouse, J. A. (2010). Insecticidal activity of scorpion toxin (ButaIT) and snowdrop lectin (GNA) containing fusion proteins towards pest species of different orders: Insecticidal activity of fusion proteins against pest species. *Pest Management Science*, *66*(1), 74–83. https://doi.org/10.1002/ps.1833.

Fitches, E. C., Pyati, P., King, G. F., & Gatehouse, J. A. (2012). Fusion to snowdrop lectin magnifies the oral activity of insecticidal ω-hexatoxin-Hv1a peptide by enabling its delivery to the central nervous system. *PLoS One*, *7*(6), e39389.

Finney, D.J. 1971. Probit analysis. Cambridge University, London, UK.

Grishin, E. V., Volkova, T., & Arseniev, A. S. (1989). Isolation and structure analysis of components from venom of the spider Argiope lobata. *Toxicon*, *27*(5), 541-549.

Kah, M., Beulke, S., Tiede, K., & Hofmann, T. (2013). Nanopesticides: state of knowledge, environmental fate, and exposure modeling. *Critical Reviews in Environmental Science and Technology*.

Kawai, N., Miwa. A., Shimazaki, K., Sahara, Y., Robinson, H. P., Nakajima, T. (1991). Spider toxin and the glutamate receptors. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 98:87-95.

Kumar, J., Ramlal, A., Mallick, D., & Mishra, V. (2021). An overview of some biopesticides and their importance in plant protection for commercial acceptance. *Plants*, *10*(6), 1185.

Lipkin, A., Kozlov, S., Nosyreva, E., Blake, A., Windass, J. D., & Grishin, E. (2002). Novel insecticidal toxins from the venom of the spider Segestria florentina. *Toxicon*, *40*(2), 125–130. https://doi.org/10.1016/S0041-0101(01)00181-7.

Mateos Fernández, R., Petek, M., Gerasymenko, I., Juteršek, M., Baebler, Š., Kallam, K., ... & Patron, N. J. (2022). Insect pest management in the age of synthetic biology. *Plant Biotechnology Journal*, *20*(1), 25-36.

Meijer, M., Dingemans, M. M., van den Berg, M., & Westerink, R. H. (2014). Inhibition of voltage-gated calcium channels as common mode of action for (mixtures of) distinct classes of insecticides. *Toxicological Sciences*, *141*(1), 103-111.

Nakasu, E. Y. T., Williamson, S. M., Edwards, M. G., Fitches, E. C., Gatehouse, J. A., Wright, G. A., & Gatehouse, A. M. R. (2014a). Novel biopesticide based on a spider venom peptide shows no adverse effects on honeybees. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1787), 20140619. https://doi.org/10.1098/rspb.2014.0619.

Nakasu, E. Y., Edwards, M. G., Fitches, E., Gatehouse, J. A., & Gatehouse, A. M. (2014b). Transgenic plants expressing ω-ACTX-Hv1a and snowdrop lectin (GNA) fusion protein show enhanced resistance to aphids. *Frontiers in plant science*, *5*, 673.

Nakasu, E. Y., Karamaouna, F., Partsinevelos, G. K., Abd El Halim, H. M., Fitches, E. C., Pyati, P., Gatehouse, J. A., Gatehouse, A. M., & Edwards, M. G. (2016). Sublethal effects of the insecticidal fusion protein *ω* -ACTX-Hv1a/GNA on the parasitoid *Eulophus pennicornis* via its host *Lacanobia oleracea*: Effects of novel insecticide on the parasitoid *E. pennicornis* via its host *L. oleracea*. *Pest Management Science*, *72*(3), 585–590. https://doi.org/10.1002/ps.4030.

Pennacchio, F., & Strand, M. R. (2006). Evolution of developmental strategies in parasitic Hymenoptera. *Annu. Rev. Entomol.*, *51*, 233-258.

Pennacchio, F., Giordana, B., & Rao, R. (2012). Applications of parasitoid virus and venom research in agriculture. In *Parasitoid Viruses* (pp. 269-283). Academic Press.

Powell, K. S., Gatehouse, A. M. R., Hilder, V. A., & Gatehouse, J. A. (1993). Antimetabolic effects of plant lectins and plant and fungal enzymes on the nymphal stages of two important rice pests, Nilaparvata lugens and Nephotettix cinciteps. *Entomologia experimentalis et applicata*, *66*(2), 119-126.

Pyati, P., Fitches, E., & Gatehouse, J. A. (2014). Optimising expression of the recombinant fusion protein biopesticide ω-hexatoxin-Hv1a/GNA in Pichia pastoris: sequence modifications and a simple method for the generation of multi-copy strains. *Journal of Industrial Microbiology and Biotechnology*, *41*(8), 1237-1247.

Rahbé, Y., Sauvion, N., Febvay, G., Peumans, W. J., & Gatehouse, A. M. (1995). Toxicity of lectins and processing of ingested proteins in the pea aphid Acyrthosiphon pisum. *Entomologia experimentalis et applicata*, *76*(2), 143-155.

Samada, L. H., & Tambunan, U. S. F. (2020). Biopesticides as promising alternatives to chemical pesticides: A review of their current and future status. *Online J. Biol. Sci*, *20*, 66-76.

Sasso, R., Gualtieri, L., Russo, E., Nugnes, F., Gebiola, M., & Bernardo, U. (2020). The establishment of a rearing technique for the fruit fly parasitoid *Baryscapus silvestrii* increases knowledge of biological, ecological and behavioural traits. *BioControl*, *65*(1), 47–57. https://doi.org/10.1007/s10526-019-09984-8.

Sauvion, N., Rahbé, Y., Peumans, W. J., Van Damme, E. J., Gatehouse, J. A., & Gatehouse, A. M. (1996). Effects of GNA and other mannose binding lectins on development and fecundity of the peach-potato aphid Myzus persicae. *Entomologia Experimentalis et Applicata*, *79*(3), 285-293.

Sukiran, N. A., Pyati, P., Willis, C. E., Brown, A. P., Readshaw, J. J., & Fitches, E. C. (2022). Enhancing the oral and topical insecticidal efficacy of a commercialized spider venom peptide biopesticide via fusion to the carrier snowdrop lectin (*Galanthus nivalis* agglutinin). *Pest Management Science*, *79*(1), 284–294. https://doi.org/10.1002/ps.7198.

Windley, M. J., Herzig, V., Dziemborowicz, S. A., Hardy, M. C., King, G. F., & Nicholson, G. M. (2012). Spider-Venom Peptides as Bioinsecticides. *Toxins*, *4*(3), Art. 3. https://doi.org/10.3390/toxins4030191.

Yang, S., Pyati, P., Fitches, E., & Gatehouse, J. A. (2014). A recombinant fusion protein containing a spider toxin specific for the insect voltage-gated sodium

ion channel shows oral toxicity towards insects of different orders. Insect Biochemistry and Molecular Biology, 47, 1–11.
Yang, S., Fitches, E., Pyati, P., & Gatehouse, J. A. (2015). Effect of insecticidal fusion proteins containing spider toxins targeting sodium and calcium ion channels on pyrethroid-resistant strains of peach-potato aphid (Myzus persicae). *Pest management science*, *71*(7), 951-956.

5 APPENDIX

PROCEEDINGS IN CONGRESS

Oral presentation

Russo E, Di Lelio I, Becchimanzi A, Shi M, Pennacchio F (2022) *Aphidius ervi* venom modulates the host-parasitoid developmental interactions. Oral presentation to the XIII Annual Meeting European PhD Network in “Insect Science” 2022, 16-18 November; Florence, Italy.

Russo E, Di Lelio I, Becchimanzi A, Shi M, Pennacchio F (2021) Host-parasitoid developmental interactions are modulated by a venom component of *Aphidius ervi* (Hymenoptera, Braconidae). Oral presentation to: XII Annual Meeting European PhD Network in “Insect Science” 2021, 17-19 November; Florence, Italy.

Russo E, Di Lelio I, Becchimanzi A, Barra E, Shi M, Giacometti R, Pennacchio F (2021) In vivo functional analysis of an *Aphidius ervi* venom protein. Oral presentation at: XXVI Congresso Nazionale Italiano di Entomologia (CNIE); 7-11 June 2021 (Virtual);

Russo E, Di Lelio I, Becchimanzi A, Shi M, Barra E, Pennacchio F (2020) In vivo functional analysis of *Aphidius ervi* venom. Oral presentation to the XI Annual Meeting European PhD Network in “Insect Science” 2020, 30/11 to 3/12, (Virtual).

Poster

Russo E, Di Lelio I, Becchimanzi A, Shi M, Pennacchio F (2022) Host-parasitoid developmental interactions are modulated by a venom component of *Aphidius ervi* (Hymenoptera, Braconidae). FISV Congress 2022. Portici, 14-16 Sett.

Russo E, Pace R, Ascolese R, Figlioli L, Miele F, Ucciero E, Nugnes F, Bernardo U (2021) Invasive quarantine species - The risk of small quantities of plant material transported by passengers. Poster presented to: XXVI Congresso Nazionale Italiano di Entomologia (CNIE); 7-11 June 2021 (Virtual).

Russo E, Nugnes F, Garonna AP, Vicinanza F, Griffo R, Bernardo U (2021) Current distribution in southern Italy and biological data on the priority pest *Aromia bungii* (Faldermann) (Coleoptera; Cerambycidae). Poster presented to: XXVI Congresso Nazionale Italiano di Entomologia (CNIE); 7-11 June 2021 (Virtual);

PUBLICATIONS

Russo E, Di Lelio I, Shi M, Becchimanzi A, Pennacchio F (2023) *Aphidius ervi* venom regulates *Buchnera* contribution to host nutritional suitability. Submitted to Journal of Insect Physiology in December 2022.

Salvatore, M. M., Di Lelio, I., DellaGreca, M., Nicoletti, R., Salvatore, F., **Russo, E**., Volpe, G., Becchimanzi, A., Mahamedi, A. E., Berraf-Tebbal, A., & Andolfi, A. (2022). Secondary Metabolites, including a New 5,6-Dihydropyran-2-One, Produced by the Fungus Diplodia corticola. Aphicidal Activity of the Main Metabolite, Sphaeropsidin A. *Molecules*, *27*(7), 2327.

Di Lelio, I., Salvatore, M. M., Della Greca, M., Mahamedi, A. E., Alves, A., Berraf-Tebbal, A., Volpe, G., **Russo, E**., Becchimanzi, A., Nicoletti, R., & Andolfi, A. (2022). Defensive Mutualism of Endophytic Fungi: Effects of Sphaeropsidin A against a Model Lepidopteran Pest. *IOCAG 2022*, 42.

Russo, E., Nugnes, F., Vicinanza, F., Garonna, A. P., & Bernardo, U. (2020). Biological and molecular characterization of *Aromia bungii* (Faldermann, 1835) (Coleoptera: Cerambycidae), an emerging pest of stone fruits in Europe. *Scientific Reports*, *10*(1), 7112.

Sasso, R., Gualtieri, L., **Russo, E**., Nugnes, F., Gebiola, M., & Bernardo, U. (2020). The establishment of a rearing technique for the fruit fly parasitoid Baryscapus silvestrii increases knowledge of biological, ecological and behavioural traits. *BioControl*, *65*(1), 47–57.

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Article

Secondary Metabolites, including a New 5,6-Dihydropyran-2-One, Produced by the Fungus *Diplodia corticola*. Aphicidal Activity of the Main Metabolite, Sphaeropsidin A

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Abstract: An undescribed 5,6-dihydropyran-2-one, namely diplopyrone C, was isolated and characterized from the cultures of an isolate of the fungus *Diplodia corticola* recovered from *Quercus suber* in Algeria. The structure and relative stereostructure of (5*S*,6*S*,7*Z*,9*S*,10*S*)-5-hydroxy-6-(2-(3-methyloxiran-2-yl)vinyl)-5,6-dihydro-2H-pyran-2-one were assigned essentially based on NMR and MS data. Furthermore, ten known compounds were isolated and identified in the same cultures. The most abundant product, the tetracyclic pimarane diterpene sphaeropsidin A, was tested for insecticidal effects against the model sucking aphid, *Acyrthosiphon pisum*. Results showed a toxic dose-dependent oral activity of sphaeropsidin A, with an LC_{50} of 9.64 mM.

Keywords: fungal metabolites; botryosphaeriaceae; metabolomics; natural products; sphaeropsidins

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1. Introduction

Diplodia (Dothideomycetes, Botryosphaeriaceae) is a widely diffused genus of fungi with more than 1000 described species [1]. Over the years, these species have been reported as pathogens or endophytes of many woody plants [1,2]. Moreover, they represent a prolific source of bioactive products with huge structural variability and bioactivities [3–6]. In this respect, the capacity of *Diplodia* spp. to have distinct habitus and interactions with plants may be related to the release of bioactive compounds during the spread in host tissues.

Among the *Diplodia* species, *Diplodia corticola* A.J.L. Phillips, A. Alves, and J. Luque is particularly regarded for the production of secondary metabolites [7–10]. It is frequently associated with dieback and canker diseases of oaks in many Mediterranean countries [11–13]. Among the metabolites frequently isolated from in vitro cultures of *D. corticola*, sphaeropsidin A is particularly promising for practical applications in agriculture and medicine due

to its exciting biological properties, including antimicrobial, insecticidal, herbicidal, and anticancer activities [14]. Besides potential applications, the documented antimicrobial and insecticidal effects of sphaeropsidin A [15–18] are relevant for further consideration of the ecological role of the fungus.

In this work, a strain of *D. corticola* isolated from *Quercus suber* in Algeria was investigated in order to increase the available data on the secondary metabolism of this fungus, leading to the isolation of a new 5,6-dihydropyran-2-one, namely diplopyrone C, and ten known compounds which include sphaeropsidin A. Following documented evidence of insecticidal properties [15,18], sphaeropsidin A was tested for aphicidal activity on the pea aphid *Acyrthosiphon pisum* (Harris) (Hemiptera, Aphididae), which is a cosmopolitan polyphagous insect and one of the primary species used as laboratory models for testing the susceptibility of sucking insects to oral administration of insecticidal products by using a feeding bioassay on an artificial diet.

2. Results

2.1. Secondary Metabolites from Cultures of Diplodia corticola B305

Crude extract obtained from the culture of *D. corticola* (strain B305), through a chromatographic purification process (see Section 4.3), gave a new metabolite, herein named diplopyrone C (**1**, Figure 1). Its structure was determined by spectroscopic methods, essentially 1D and 2D NMR, IR, and UV combined with mass spectrometry, as reported below (Figures S1–S8). Moreover, ten known metabolites were identified by comparison of their proton spectra (Figures S9–S18), and eventually optical rotation, with those reported in the literature for: sphaeropsidins A and B (**2** and **3**, [19]), sphaeropsidin C (**4**) [20], (*R*)-mellein, (3*R*,4*R*)- and (3*R*,4*S*)-4-hydroxymelleins (**5**–**7**) [21], sapinofuranone B (**8**) [22], pinofuranoxin A (**9**) [23], diplobifuranylone B (**10**) [24], and tyrosol (**11**) [25] (Figure 1).

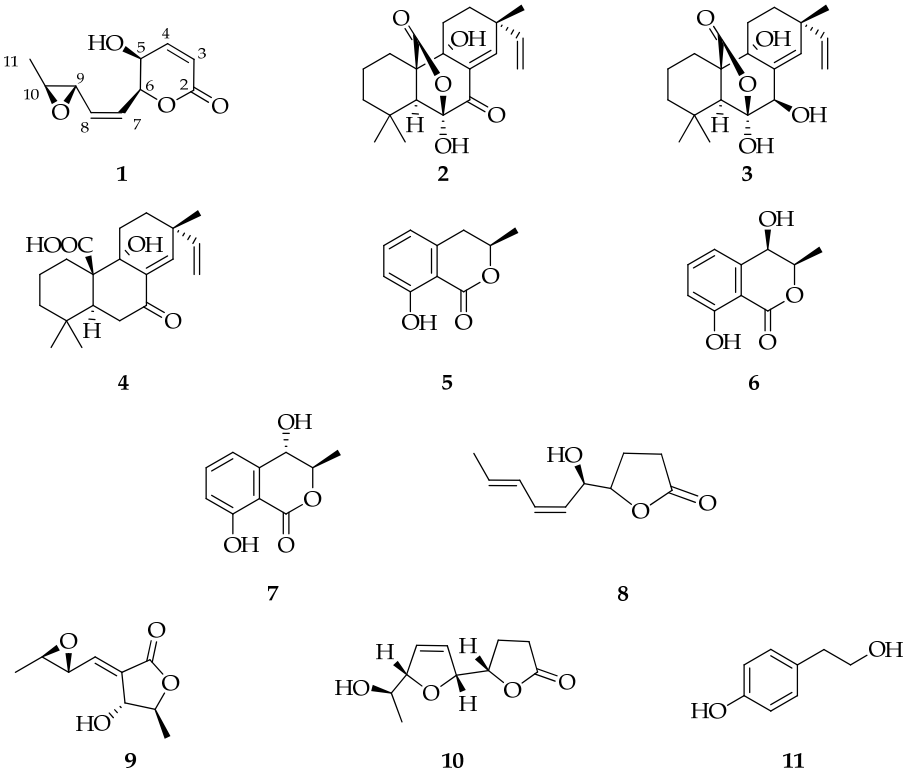

Figure 1. Structures of diplopyrone C (**1**), sphaeropsidins A (**2**), B (**3**), and C (**4**), (3*R*)-mellein (**5**), (3*R*,4*R*)-(**6**), and, (3*R*,4*S*)-4-hydroxymelleins (**7**), sapinofuranone B (**8**), pinofuranoxin A (**9**), diplobifuranylone B (**10**), and tyrosol (**11**).

The HRESI-MS of compound **1** showed picks at m/z = 235.0395 $[M + K]^+$, and 219.1860 $[M + Na]^+$ suggesting the molecular formula $C_{10}H_{12}O_4$ and five degrees of unsaturation. Moreover, fragment ion at m/z = 179.0721 $[M\text{-}OH]^+$ was evident (Figure S8).

^1H and ^{13}C NMR analysis showed signals typical of 5,6-disubstitute-5,6-dihydropyran-2-one (Figures S1 and S2) [26] in agreement with IR and UV spectra. In fact, the ^1H NMR spectrum showed signals at δ (*J* in Hz): 7.03 (dd, 9.8, 5.4), 6.17 (d, 9.8) 5.40 (dd, 7.9, 3.3), and 4.28 (dd, 5.4, 3.3) assigned to H-4, H-3, H-6 and H-5 of 5,6-dihydropyran-2-one ring (Table 1). The COSY spectrum (Figure S3) confirmed this hypothesis, and chemical shifts H-3 to H-6 protons were assigned. In the ^{13}C NMR spectrum (Figure S2), signals at δ 162.8, 122.9, 144.3, and 76.9 confirmed the presence of an α,β-unsaturated lactone. Moreover, the signal at δ 4.28 (H-5) correlated to the carbon at δ 63.2 in the HSQC spectrum (Figure S4), indicating the presence of the hydroxyl group at C-5. The remaining signals observed in the 1H NMR spectrum at δ (*J* in Hz): 5.93 (ddd, 11.7, 7.9, 1.1), 5.63 (ddd, 11.7, 6.7, 1.1), 3.34 (dd, 5.2, 2.1 H), 3.00 (dq, 5.2, 2.1 Hz), and 1.40 (d, 5.2) were correlated, in the HSQC spectrum, to δ 127.2, 132.4, 55.5, 56.7, and 17.5, respectively. Analysis of the HMBC spectrum (Figure S5) showed correlations between H-6 and C-7 and C-8, and H-8 with C-9 and C-10 indicated a 3,4-oxirane-1-pentenyl side chain at C-6 (Figure 2).

Table 1. NMR data of diplopyrone C (**1**) in $CDCl_3$ [1,2].

No.	δC	δH (*J* in Hz)	HMBC
1	-	-	
2	162.8	-	
3	122.9	6.17 (1H) br d (9.8)	C-2, C-5
4	144.3	7.03 (1H) dd (9.8, 5.4)	C-2, C-5, C-6
5	63.2	4.28 (1H) dd (5.4, 3.3)	C-3, C-4, C-6
6	76.9	5.40 (1H) dd (7.9, 3.3)	C-7, C-8
7	127.2	5.93 (1H) ddd (11.7, 7.9, 1.1)	C-9
8	132.4	5.63 (1H) ddd (11.7, 6.7, 1.1)	C-6, C-9, C-10
9	55.5	3.34 (1H) dd (6.7, 2.1)	C-8, C-7, C-10
10	56.7	3.00 (1H) dq (5.2, 2.1)	C-11
11	17.5	1.40 (3H) d (5.2)	C-9, C-8

[1] The chemical shifts are in δ values (ppm). [2] 2D ^1H, ^1H (COSY) ^{13}C, and ^1H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons. Numbering is according to that in Figure 1.

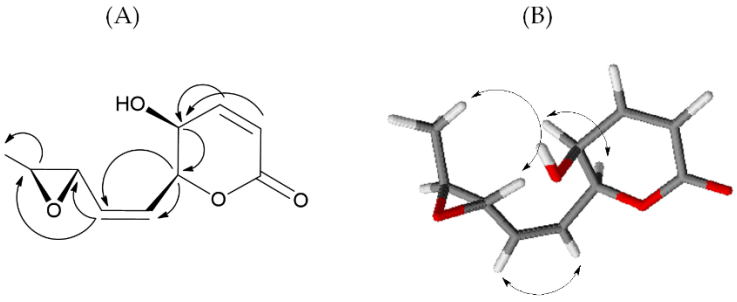

Figure 2. Significant HMBC (**A**) and NOESY (**B**) correlations of **1**.

The *Z* configuration of the double bond at C-7-C-8 was assigned on the basis of the typical coupling constant (11.7 Hz) [27]. The relative configuration of compound **1** was assigned on the basis of the NOE effects observed in the NOESY spectrum (Figure S6). The NOE effect of H-7 with H-8 confirmed the *Z* configuration of the chain double bond. The NOE effect of H-5 with H-6 and the coupling constant of 3.3 Hz indicated a *cis* configuration of the hydroxyl group and the side chain of the dihydropyrone ring. The absence of

the NOE effect between the H-9 and the H-10 and the NOE effect of the H-11 methyl with the H-9 indicated a *trans* configuration of the oxirane ring (Figure 2). All spectral data allowed to the determine the structure and relative stereostructure of compound **1** as (5*S*,6S,7*Z*,9*S*,10*S*)-5-hydroxy-6-(2-(3-methyloxiran-2-yl)vinyl)-5,6-dihydro-2H-pyran-2-one, named diplopyrone C.

2.2. Oral Toxicity of Sphaeropsidin A on Acyrthosiphon pisum

Sphaeropsidin A showed an oral lethal activity on aphids at all the doses tested, and the resulting survival rate was significantly lower compared to the control (log-rank test: $\chi^2 = 561.1$, $p < 0.0001$, dF = 4). The highest doses induced mortality starting from day 2. The mortality increased over time in a dose-dependent manner, and no aphids survived after 7 days of administration (Figure 3).

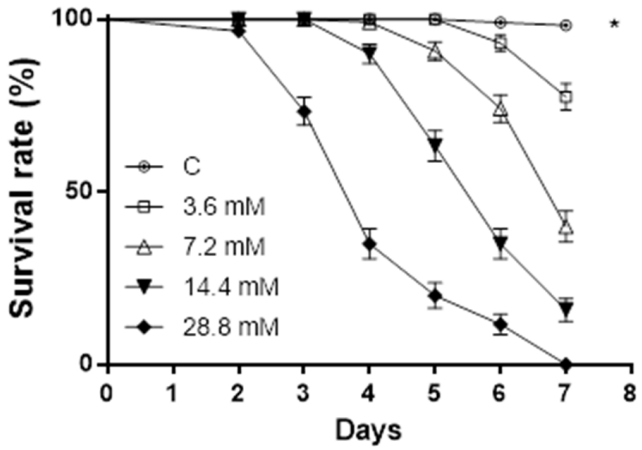

Figure 3. Sphaeropsidin A oral toxicity on *A. pisum*. Aphids' survival rate was negatively affected by sphaeropsidin A oral administration. Asterisk indicates a statistical difference to log-rank (Mantel–Cox) test ($p < 0.0001$).

The lethal concentrations of sphaeropsidin A, resulting in 10%, 50%, and 90% mortality of the pea aphids (LC_{10}, LC_{50}, and LC_{90}, respectively) and 95% confidence intervals, were determined on day 6 and showed that LC_{10} (95% CI) is 4.5 mM (4.12–4.93), LC_{50} (95% CI) is 9.64 mM (9.23–10.06), and LC_{90} is 20.43 mM (18.81–21.57). The lethal activity of sphaeropsidin A on day 6 is shown in Figure 4.

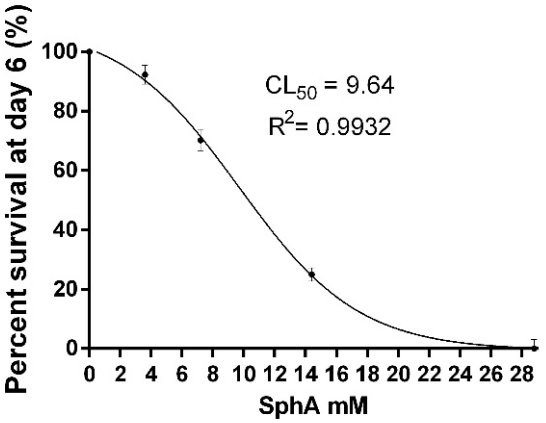

Figure 4. The dose-dependent survival rate of aphids exposed to sphaeropsidin A. Values are reported as mean ± standard deviation (SD) of three replicates in four separate experiments and expressed as a percentage of the control aphids.

3. Discussion

Secondary metabolites are often used by microbes to enable unique trophic lifestyles, overcome competition with other microbes, or cope with environmental biotic and abiotic stress [28–31]. Hence, in this study, the production of secondary metabolites from a strain of *D. corticola* was examined with reference to its possible ecological role.

The new 5,6-dihydropyran-2-one, namely diplopyrone C, and several known metabolites were isolated and identified from the fungal strain under examination. This metabolite is closely related to diplopyrone B, which was recently isolated from the same fungal species associated with *Q. suber* in Sardinia (Italy) and characterized as the 5-hydroxy-6-(penta-1,3-dienyl)-5,6-dihydro-pyran-2-one [7]. The 6-substituted derivatives of 5,6-dihydropyran-2-ones (or 5,6-dihydro-α-pyrones) are polyketides produced by several microorganisms and plants. Many of these products are biologically active, exhibiting phytotoxicity, cytotoxicity against tumor cells, and antimicrobial activity [32,33].

Moreover, accurate screening of the existing literature showed that a number of metabolites identified in this study were previously reported as products of *Diplodia* species. In particular, sphaeropsidins A–C (**2**–**4**), (3*R*,4*R*)- and (3*S*,4*R*)-hydroxymelleins (**6**,**7**), sapinofuranone B (**8**), and diplobifuranylone B (**10**) were already identified from cultures of *D. coriticola* (Table 2).

Table 2. Secondary metabolites identified in this work and previously reported as products of *Diplodia* spp.

Code	Name	Source	Ref.
2	Sphaeropsidin A	*D. corticola*, *D. sapinea*, *D. africana*, *D. quercivora*	[3,10,34–37]
3	Sphaeropsidin B	*D. corticola*, *D. sapinea*	[10,34,37]
4	Sphaeropsidin C	*D. corticola*, *D. sapinea*, *D. quercivora*	[10,34,36,37]
5	(*R*)-mellein	*D. africana*, *D. fraxini*, *D. mutila*, *D. seriata*, *D. sapinea*	[3,4,21,38]
6	(3*R*,4*R*)-4-hydroxymellein	*D. corticola*, *D. africana*, *D. sapinea*	[3,10,21]
7	(3*S*,4*R*)-4-hydroxymellein	*D. corticola*, *D. africana*, *D. sapinea*	[3,10,21]
8	Sapinofuranone B	*D. corticola*	[10]
9	Pinofuranoxin A	*D. sapinea*	[23]
10	Diplobifuranylone B	*D. corticola*	[10]
11	Tyrosol	*D. fraxini*, *D. mutila*	[4,38]

(*R*)-Mellein, (3*R*,4*R*)- and (3*S*,4*R*)-hydroxymelleins (**5**–**7**) belong to the group of 3,4-dihydroisocoumarins, also known as melleins, which are lactonic natural products [39] commonly produced in vitro by botryosphaeraceous fungi, such as *Lasiodiplodia* spp., *Macrophomina* spp., and *Neofusicoccum* spp. [40–42]. Furthermore, (*R*)-mellein is considered a vivotoxin since it was also isolated from plants inoculated with the mycelium of *Neofusicoccum parvum* [43].

The high production rate of sphaeropsidin A suggests its possible involvement in the dynamic interaction with the host plant; in this regard, the anti-insectan properties deserve particular attention in regard to the hypothesis that this compound is also released in vivo. In fact, a widespread anti-insectan effect is corroborated by results of previous studies showing fagodeterrent and larvicidal activity against the mosquito *Aedes aegypti* (Diptera, Culicidae) [15] and oral toxic activity against larvae of the chewing model insect *Spodoptera littoralis* (Lepidoptera, Noctuidae) [18].

Here we have demonstrated the possible effects of sphaeropsidin A on sucking insects based on a dose-dependent toxic oral activity against the model phloem sucking insect,

A. pisum. Hence, if confirmed in planta, production of this secondary metabolite may reduce the impact of herbivorous insects, representing an indication of defensive mutualism established during the development of this fungus as an endophyte or latent pathogen.

Moreover, the oral toxic activity shown by sphaeropsidin A on pea aphids stimulates further investigation of its mode of action from the perspective of its possible application as a new pesticide that meets the growing demand for alternative products with low environmental impact.

4. Materials and Methods

4.1. General Experimental Procedures

The optical rotations of pure metabolites were measured in $CHCl_3$ or MeOH on a Jasco P-1010 digital polarimeter (Tokyo, Japan). FT–IR spectra were recorded in modality ATR (attenuated total reflectance) with model Nicolet 5700 by Thermo Electric Corporation (Waltham, MA, USA). The measuring cell consisted of a mono crystal of zinc selenide. The blank was recorded using air as reference. UV spectra were recorded in CH_3CN by Cary model 5000 Spectrophotometer by Varian C. (Palo Alto, CA, USA). ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX instrument at 400 and 100 MHz, respectively, in $CDCl_3$. The same solvents were used as internal standards. COSY-45, HSQC, HMBC, and NOESY were performed using standard Bruker microprograms. TLC was performed on silica gel (Kieselgel 60, F_{254}, 0.25 mm, Merck, Darmstadt, Germany) or reverse-phase plates (Whatman, KC18 F_{254}, 0.20 mm). The spots were visualized by exposure to UV radiation (253 nm) or by spraying first with 10% H_2SO_4 in methanol followed by heating at 110 °C for 10 min. Chromatography was performed on silica gel column (Merck, Kieselgel 60, 0.063–0.200 mm). HRESI-TOF mass spectra were measured on an Agilent Technologies ESI-TOF 6230DA instrument in the positive ion mode (Milan, Italy).

4.2. Fungal Strain and Cultures Production

Diplodia corticola strain (B305) employed in this study was previously isolated from *Q. suber* trees showing canker and dieback symptoms in Algeria. The strain was identified and characterized as a pathogen in a previous work [11] based on the integration of morphological features and phylogenetic analysis of the combined ITS and *tef1-α* sequence data. The nucleotide sequences of *D. corticola* are available in GenBank database under accession numbers MT015626 and MT066136.

Liquid cultures of the strain were prepared in Czapek-Dox broth (Oxoid, Thermo Scientific, Waltham, MA, USA) amended with 2% cornmeal in 500 mL Erlenmeyer flasks containing 250 mL of the substrate [44] and grown in a stationary phase in the dark at 25 °C for 30 days.

4.3. Extraction and Purification Processes of Metabolites 1–11

The culture broth and mycelia were homogenized in a mixer with 350 mL of MeOH (1% NaCl). Then, the suspension was centrifuged for 40 min at 7000 rpm and 10 °C. The pellet was resuspended in 150 mL of a mixture of H_2O:MeOH (45:55 *v*/*v*, 1% NaCl) and submitted to a second homogenization followed by centrifugation. Supernatants were collected, and MeOH was evaporated under reduced pressure to obtain an aqueous solution for the subsequent extraction (3 times) with ethyl acetate at native pH (=6.0). The organic phases were combined, dried with anhydrous Na_2SO_4, and evaporated under reduced pressure, yielding crude extract as brown oil (156.7 mg). The organic extract was purified by column chromatography (CC) on silica gel (40 cm × 1.5 cm i.d.) eluted with $CHCl_3$/*i*-PrOH (95:5, *v*/*v*), originating 8 homogeneous fractions (A 3.7 mg, B 6.7 mg, C 43.3 mg, D 15.2 mg, E 9.1 mg, F 15.9 mg, G 2.3 mg, H 32.4 mg), the last of which was collected by eluting with methanol. Fraction C was purified by TLC on silica gel eluted with *n*-hexane/EtOAc (6:4, *v*/*v*) to give **2** (35.4 mg, white crystalline solid, R_f 0.59), **4** (2.0 mg white solid R_f 0.50), and **5** (1.2 mg yellowing oil, R_f 0.79). Fraction D was purified by TLC on silica gel eluted with *n*-hexane/EtOAc (1:1, *v*/*v*) to give **3** (5.2 mg, white crystalline solid, R_f 0.76), and a mixture

of **6** and **7**, which was separated by reversed-phase TLC using H_2O-EtOH (1:1, *v/v*) (2.5 and 3.1 mg, white amorphous solids, R_f 0.54 and 0.58, respectively), **9** (2.1 mg, homogeneous oil, R_f 0.48), and **8** (1.3 mg, yellowing oil, R_f 0.45). Fraction F was purified by TLC on silica gel eluted with $CHCl_3$/*i*-PrOH (95:5, *v/v*), giving **10** (7.8 mg as colorless oil, R_f 0.42), **11** (1.5 mg as white amorphous solid, R_f 0.39), and **1** (5.4 mg, yellowing amorphous solid, R_f 0.37).

Diplopyrone C (**1**): yellowing amorphous solid; $[\alpha]^{25}_D$ +36 (c 0.24); IR ν_{max}: 3433, 1720, 1629, 1373, 1254, 1221 cm^{-1}; UV λ_{max} nm (log ε) 203 (2.87); ^1H and ^{13}C NMR spectra: see Table 1; HR-ESIMS (+) *m/z*: 235.0395 [calcd. for $C_{10}H_{12}KO_4$ 235.0373, M + K]$^+$, 219.1860 [calcd. for $C_{10}H_{12}NaO_4$ 219.1896, M + Na]$^+$, 179.0721 [calcd. for $C_{10}H_{11}O_3$ 179.0708 M-OH]$^+$.

4.4. Insects Rearing and Oral Toxicity Bioassay

Acyrthosiphon pisum was reared on potted broad bean plants (*Vicia faba*) at 20 ± 1 °C, 75 ± 5% RH, and under a 16:8 h light:dark photoperiod, starting with insects originally collected from alfalfa plants in Eboli, southern Italy. In order to synchronize the aphid population, parthenogenetic adult females were placed on plants for 6 h, resulting in neonate nymphs with an age of 0–6 h that were used throughout the experiments.

The oral toxicity of sphaeropsidin A (**2**) on *A. pisum* was investigated using a standard basal diet previously developed for assays of test compounds [45]. The feeding system for the pea aphid was realized as described in [45] with minor modifications. Each experimental unit was a feeding system with 10 aphids; four replications per treatment were realized, each replicate consisting of three experimental units per treatment. A total of 120 aphids per treatment were used. In each feeding system, 300 µL of artificial diet containing **1** 3.6 mM, 7.2 mM, 14.4 mM, and 28.8 mM was dispensed; negative control was realized using the artificial diet only. The experiment was carried out under the rearing conditions described above. Briefly, on day 0, neonate nymphs were transferred to a freshly prepared diet sachet feeding apparatus. Mortality was recorded daily for one week, and dead nymphs were removed. The artificial diet was replaced every two days. The lethal concentrations of **1** resulting in 10%, 50%, and 90% aphid mortality (defined as LC_{10}, LC_{50}, and LC_{90}) and the corresponding 95% confidence intervals were determined.

4.5. Statistical Analysis

Aphid survival curves were compared using Kaplan–Meier and log-rank analysis. The results obtained were analyzed using non-linear sigmoid curve fitting, and the activity of each treatment was evaluated on day 6 on the basis of dose–response concentrations; the goodness of fit to the curve model was evaluated on the basis of R^2 values. Data were analyzed using Prism 6 (GraphPad Software Inc. version 6.0b, San Diego, CA, USA).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27072327/s1, Figure S1: ^1H NMR spectrum of diplopyrone C (**1**) recorded in $CDCl_3$ at 400 MHz; Figure S2: ^{13}C NMR spectrum of diplopyrone (**1**) recorded in $CDCl_3$ at 100 MHz; Figure S3: ^1H,^1H COSY spectrum of diplopyrone C (**1**) recorded in $CDCl_3$ at 400 MHz; Figure S4: HSQC spectrum of diplopyrone C (**1**) recorded in $CDCl_3$ at 400 MHz; Figure S5: HMBC spectrum of diplopyrone C (**1**) recorded in $CDCl_3$ at 400 MHz; Figure S6: NOESY spectrum of diplopyrone C (1) recorded in $CDCl_3$ at 400 MHz; Figure S7: IR spectrum of diplopyrone C (**1**); Figure S8: HRESI MS spectrum of diplopyrone C (**1**) recorded in positive mode; Figure S9: ^1H NMR spectrum of sphaeropsidin A (**2**) recorded in $CDCl_3$ at 400 MHz; Figure S10: ^1H NMR spectrum of sphaeropsidin B (**3**) recorded in $CDCl_3$ at 400 MHz; Figure S11: ^1H NMR spectrum of sphaeropsidin C (**4**) recorded in $CDCl_3$ at 400 MHz; Figure S12: ^1H NMR spectrum of (3*R*)-mellein (**5**) recorded at 400 MHz in CDCl3; Figure S13: ^1H NMR spectrum of (3*R*,4*R*)-4-hydroxymellein (**6**) recorded at 400 MHz in $CDCl_3$; Figure S14: ^1H NMR spectrum of (3*R*,4*S*)-4-hydroxymellein (**7**) recorded at 400 MHz in $CDCl_3$; Figure S15: ^1H NMR spectrum sapinofuranone B (**8**) recorded at 400 MHz in $CDCl_3$; Figure S16: ^1H NMR spectrum of pinofuranoxin A (**9**) recorded at 400 MHz in $CDCl_3$; Figure S17: ^1H NMR spectrum of diplobifuranylone B (**10**) recorded at 400 MHz in $CDCl_3$; Figure S18: ^1H NMR spectrum tyrosol (**11**) recorded at 400 MHz in $CDCl_3$.

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References

1. Phillips, A.J.L.; Lopes, J.; Abdollahzadeh, J.; Bobev, S.; Alves, A. Resolving the *Diplodia* complex on apple and other *Rosaceae* hosts. *Pers. Mol. Phylogeny Evol. Fungi* **2012**, *29*, 29–38. [CrossRef] [PubMed]
2. Slippers, B.; Wingfield, M.J. Botryosphaeriaceae as endophytes and latent pathogens of woody plants: Diversity, ecology and impact. *Fungal Biol. Rev.* **2007**, *21*, 90–106. [CrossRef]
3. Evidente, A.; Masi, M.; Linaldeddu, B.T.; Franceschini, A.; Scanu, B.; Cimmino, A.; Andolfi, A.; Motta, A.; Maddau, L. Afritoxinones A and B, dihydrofuropyran-2-ones produced by *Diplodia africana* the causal agent of branch dieback on *Juniperus phoenicea*. *Phytochemistry* **2012**, *77*, 245–250. [CrossRef] [PubMed]
4. Reveglia, P.; Savocchia, S.; Billones-Baaijens, R.; Masi, M.; Cimmino, A.; Evidente, A. Phytotoxic metabolites by nine species of Botryosphaeriaceae involved in grapevine dieback in Australia and identification of those produced by *Diplodia mutila*, *Diplodia seriata*, *Neofusicoccum australe* and *Neofusicoccum luteum*. *Nat. Prod. Res.* **2019**, *33*, 2223–2229. [CrossRef]
5. Liu, X.Y.; Wang, X.L.; Shen, T.; Ren, D.M.; Lou, H.X.; Wang, X.N. Two new triterpenoids from the fungus *Diplodia cupressi*. *Nat. Prod. Res.* **2020**, *34*, 2179–2185. [CrossRef]
6. Tuzi, A.; Andolfi, A.; Maddau, L.; Masi, M.; Evidente, A. Structure and stereochemical assignment of spheropsidone, a phytotoxin from *Diplodia cupressi*. *J. Struct. Chem.* **2012**, *53*, 786–792. [CrossRef]
7. Masi, M.; Maddau, L.; Linaldeddu, B.T.; Cimmino, A.; D'Amico, W.; Scanu, B.; Evidente, M.; Tuzi, A.; Evidente, A. Bioactive secondary metabolites produced by the oak pathogen *Diplodia corticola*. *J. Agric. Food Chem.* **2016**, *64*, 217–225. [CrossRef]
8. Di Lecce, R.; Masi, M.; Linaldeddu, B.T.; Pescitelli, G.; Maddau, L.; Evidente, A. Bioactive secondary metabolites produced by the emerging pathogen *Diplodia olivarum*. *Phytopathol. Mediterr.* **2021**, *60*, 129–138. [CrossRef]
9. Cimmino, A.; Maddau, L.; Masi, M.; Evidente, M.; Linaldeddu, B.T.; Evidente, A. Further secondary metabolites produced by *Diplodia corticola*, a fungal pathogen involved in cork oak decline. *Tetrahedron* **2016**, *72*, 6788–6793. [CrossRef]
10. Evidente, A.; Andolfi, A.; Fiore, M.; Spanu, E.; Franceschini, A.; Maddau, L. Diplobifuranylones A and B, 5′-monosubstituted tetrahydro-2H-bifuranyl-5-ones produced by *Diplodia corticola*, a fungus pathogen of cork oak. *J. Nat. Prod.* **2006**, *69*, 671–674. [CrossRef]
11. Mahamedi, A.E.; Phillips, A.J.L.; Lopes, A.; Djellid, Y.; Arkam, M.; Eichmeier, A.; Zitouni, A.; Alves, A.; Berraf-Tebbal, A. Diversity, distribution and host association of Botryosphaeriaceae species causing oak decline across different forest ecosystems in Algeria. *Eur. J. Plant Pathol.* **2020**, *158*, 745–765. [CrossRef]
12. Félix, C.; Pinto, G.; Amaral, J.; Fernandes, I.; Alves, A.; Esteves, A.C. Strain-related pathogenicity in *Diplodia corticola*. *For. Pathol.* **2017**, *47*, e12366. [CrossRef]
13. Linaldeddu, B.T.; Maddau, L.; Franceschini, A. First report of *Diplodia corticola* causing canker and dieback of *Quercus ilex*, *Q. petraea*, and *Q. suber* in Corsica (France). *Plant Dis.* **2017**, *101*, 256. [CrossRef]
14. Masi, M.; Evidente, A. Sphaeropsidin A: A pimarane diterpene with interesting biological activities and promising practical applications. *ChemBioChem* **2021**, *22*, 3263–3269. [CrossRef] [PubMed]
15. Cimmino, A.; Andolfi, A.; Avolio, F.; Ali, A.; Tabanca, N.; Khan, I.A.; Evidente, A. Cyclopaldic acid, seiridin, and sphaeropsidin A as fungal phytotoxins, and larvicidal and biting deterrents against *Aedes aegypti* (Diptera: Culicidae): Structure-activity relationships. *Chem. Biodivers.* **2013**, *10*, 1239. [CrossRef]
16. Evidente, A.; Venturi, V.; Masi, M.; Degrassi, G.; Cimmino, A.; Maddau, L.; Andolfi, A. In vitro antibacterial activity of sphaeropsidins and chemical derivatives toward *Xanthomonas oryzae* pv. *oryzae*, the causal agent of rice bacterial blight. *J. Nat. Prod.* **2011**, *74*, 2520–2525. [CrossRef]
17. Padhi, S.; Masi, M.; Cimmino, A.; Tuzi, A.; Jena, S.; Tayung, K.; Evidente, A. Funiculosone, a substituted dihydroxanthene-1,9-dione with two of its analogues produced by an endolichenic fungus *Talaromyces funiculosus* and their antimicrobial activity. *Phytochemistry* **2019**, *157*, 175–183. [CrossRef]
18. Di Lelio, I.; Salvatore, M.M.; DellaGreca, M.; Mahamedi, A.E.; Alves, A.; Berraf-Tebbal, A.; Volpe, G.; Russo, E.; Becchimanzi, A.; Nicoletti, R. Defensive mutualism of endophytic fungi: Effects of sphaeropsidin A against a model lepidopteran pest. *Proceeding Pap.* **2022**. [CrossRef]
19. Ellestad, G.A.; Kunstmann, M.P.; Mirando, P.; Morton, G.O. Structures of fungal diterpene antibiotics LL-S491/3 and -y. *J. Am. Chem. Soc.* **1972**, *236*, 6206–6208. [CrossRef]

20. Evidente, A.; Sparapano, L.; Fierro, O.; Bruno, G.; Giordano, F.; Motta, A. Sphaeropsidins B and C, phytotoxic pimarane diterpenes from *Sphaeropsis sapinea* f. Sp. *Cupressi Diplodia Mutila*. *Phytochemistry* **1997**, *45*, 705–713. [CrossRef]
21. Cabras, A.; Mannoni, M.A.; Serra, S.; Andolfi, A.; Fiore, M.; Evidente, A. Occurrence, isolation and biological activity of phytotoxic metabolites produced in vitro by *Sphaeropsis sapinea*, pathogenic fungus of *Pinus radiata*. *Eur. J. Plant Pathol.* **2006**, *115*, 187–193. [CrossRef]
22. Evidente, A.; Sparapano, L.; Fierro, O.; Bruno, G.; Motta, A. Sapinofuranones A and B, two new 2(3H)-dihydrofuranones produced by *Sphaeropsis sapinea*, a common pathogen of conifers. *J. Nat. Prod.* **1999**, *62*, 253–256. [CrossRef] [PubMed]
23. Masi, M.; Di Lecce, R.; Marsico, G.; Linaldeddu, B.T.; Maddau, L.; Superchi, S.; Evidente, A. Pinofuranoxins A and B, bioactive trisubstituted furanones produced by the invasive pathogen *Diplodia sapinea*. *J. Nat. Prod.* **2021**, *84*, 2600–2605. [CrossRef] [PubMed]
24. Cheng, X.; Quintanilla, C.D.; Zhang, L. Total synthesis and structure revision of diplobifuranylone B. *J. Org. Chem.* **2019**, *84*, 11054–11060. [CrossRef] [PubMed]
25. Félix, C.; Salvatore, M.M.; DellaGreca, M.; Ferreira, V.; Duarte, A.S.; Salvatore, F.; Naviglio, D.; Gallo, M.; Alves, A.; Esteves, A.C.; et al. Secondary metabolites produced by grapevine strains of *Lasiodiplodia theobromae* grown at two different temperatures. *Mycologia* **2019**, *111*, 466–476. [CrossRef]
26. D'Annibale, A.; Ciaralli, L.; Bassetti, M.; Pasquini, C. Synthesis of alkyl-substituted six-membered lactones through ring-closing metathesis of homoallyl acrylates. An easy route to pyran-2-ones, constituents of tobacco flavor. *J. Org. Chem.* **2007**, *72*, 6067–6074. [CrossRef]
27. Badertscher, M.; Bühlmann, P.; Pretsch, E. *Structure Determination of Organic Compounds*; Springer: Berlin/Heidelberg, Germany, 2009; ISBN 978-3-540-93809-5.
28. Salvatore, M.M.; Andolfi, A.; Nicoletti, R. The thin line between pathogenicity and endophytism: The case of *Lasiodiplodia theobromae*. *Agriculture* **2020**, *10*, 488. [CrossRef]
29. Félix, C.; Salvatore, M.M.; Dellagreca, M.; Meneses, R.; Duarte, A.S.; Salvatore, F.; Naviglio, D.; Gallo, M.; Jorrín-Novo, J.V.; Alves, A.; et al. Production of toxic metabolites by two strains of *Lasiodiplodia theobromae*, isolated from a coconut tree and a human patient. *Mycologia* **2018**, *110*, 642–653. [CrossRef]
30. Shabuer, G.; Ishida, K.; Pidot, S.J.; Roth, M.; Dahse, H.M.; Hertweck, C. Plant pathogenic anaerobic bacteria use aromatic polyketides to access aerobic territory. *Science* **2015**, *350*, 670–674. [CrossRef]
31. Salvatore, M.M.; Andolfi, A. Phytopathogenic fungi and toxicity. *Toxins* **2021**, *13*, 689. [CrossRef]
32. Davies-Coleman, M.T.; Rivett, D.E.A. Naturally occurring 6-substituted 5, 6-dihydro-α-pyrones. In *Fortschritte der Chemie Organischer Naturstoffe/Progress in the Chemistry of Organic Natural Products*; Springer: Vienna, Austria, 1998; pp. 181–209, ISBN 978-3-7091-9004-3.
33. Dickinson, J.M. Microbial pyran-2-ones and dihydropyran-2-ones. *Nat. Prod. Rep.* **1991**, *10*, 71–98. [CrossRef] [PubMed]
34. Sparapano, L.; Bruno, G.; Fierro, O.; Evidente, A. Studies on structure-activity relationship of sphaeropsidins A-F, phytotoxins produced by *Sphaeropsis sapinea* f. sp. *cupressi*. *Phytochemistry* **2004**, *65*, 189–198. [CrossRef] [PubMed]
35. Evidente, A.; Sparapano, L.; Motta, A.; Giordano, F.; Fierro, O.; Frisullo, S. A phytotoxic pimarane diterpene of *Sphaeropsis sapinea* f. sp. *cupressi*, the pathogen of a canker disease of cypress. *Phytochemistry* **1996**, *42*, 1541–1546. [CrossRef]
36. Andolfi, A.; Maddau, L.; Basso, S.; Linaldeddu, B.T.; Cimmino, A.; Scanu, B.; Deidda, A.; Tuzi, A.; Evidente, A. Diplopimarane, a 20-nor-ent-pimarane produced by the oak pathogen *Diplodia quercivora*. *J. Nat. Prod.* **2014**, *77*, 2352–2360. [CrossRef]
37. Evidente, A.; Sparapano, L.; Andolfi, A.; Bruno, G.; Giordano, F.; Motta, A. Chlorosphaeropsidone and epichlorosphaeropsidone, two new chlorinated dimedone methyl ethers isolated from liquid cultures of *Sphaeropsis sapinea* f. sp. *cupressi*. *Phytopathol. Mediterr.* **2000**, *39*, 299–330.
38. Cimmino, A.; Maddau, L.; Masi, M.; Linaldeddu, B.T.; Pescitelli, G.; Evidente, A. Fraxitoxin, a new isochromanone isolated from *Diplodia fraxini*. *Chem. Biodivers.* **2017**, *14*, e1700325. [CrossRef]
39. Saeed, A. Isocoumarins, miraculous natural products blessed with diverse pharmacological activities. *Eur. J. Med. Chem.* **2016**, *116*, 290–317. [CrossRef]
40. Salvatore, M.M.; Alves, A.; Andolfi, A. Secondary metabolites produced by *Neofusicoccum* species associated with plants: A review. *Agriculture* **2021**, *11*, 149. [CrossRef]
41. Salvatore, M.M.; Alves, A.; Andolfi, A. Secondary metabolites of *Lasiodiplodia theobromae*: Distribution, chemical diversity, bioactivity, and implications of their occurrence. *Toxins* **2020**, *12*, 457. [CrossRef]
42. Salvatore, M.M.; Félix, C.; Lima, F.; Ferreira, V.; Naviglio, D.; Salvatore, F.; Duarte, A.S.; Alves, A.; Andolfi, A.; Esteves, A.C. Secondary metabolites produced by *Macrophomina phaseolina* isolated from *Eucalyptus globulus*. *Agriculture* **2020**, *10*, 72. [CrossRef]
43. Abou-Mansour, E.; Débieux, J.L.; Ramírez-Suero, M.; Bénard-Gellon, M.; Magnin-Robert, M.; Spagnolo, A.; Chong, J.; Farine, S.; Bertsch, C.; L'Haridon, F.; et al. Phytotoxic metabolites from *Neofusicoccum parvum*, a pathogen of Botryosphaeria dieback of grapevine. *Phytochemistry* **2015**, *115*, 207–215. [CrossRef] [PubMed]
44. Salvatore, M.M.; DellaGreca, M.; Nicoletti, R.; Salvatore, F.; Tuzi, A.; De Tommaso, G.; Alves, A.; Mahamedi, A.E.; Berraf-Tebbal, A.; Andolfi, A. Mitidjospirone, a new spirodioxynaphthalene and GC-MS screening of secondary metabolites produced by strains of *Lasiodiplodia mitidjana* associated to *Citrus sinensis* dieback. *Nat. Prod. Res.* **2021**, 1–10. [CrossRef] [PubMed]
45. Sadeghi, A.; Van Damme, E.J.M.; Smagghe, G. Evaluation of the susceptibility of the pea aphid, *Acyrthosiphon pisum*, to a selection of novel biorational insecticides using an artificial diet. *J. Insect Sci.* **2009**, *9*, 65. [CrossRef] [PubMed]

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Defensive Mutualism of Endophytic Fungi: Effects of Sphaeropsidin A against a Model Lepidopteran Pest †

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Abstract: Sphaeropsidin A (SphA) is a pimarane diterpene produced by several fungi associated with plants. Following previous evidence of insecticidal properties of SphA, we investigated its contact and oral toxicity against the model chewing lepidopteran *Spodoptera littoralis*. The compound showed no lethal effect when directly sprayed on larvae, while it produced an evident oral toxic effect, associated with sublethal effects. These results demonstrated that SphA might play a defensive role against lepidopteran insects in plants harboring the producing fungus, depending on the extent at which the endophytic strains are able to perform biosynthesis of this and eventually other bioactive metabolites in vivo.

Keywords: endophytic fungi; secondary metabolites; oral toxicity; lepidopteran pests

1. Introduction

Microbiome associated to plants is more and more regarded as a basic factor regulating their fitness, with reference to the effects of the mutual interactions among and between the holobiont constituents [1–3]. In most instances, symbiotic relationships between fungi and plants are considered with reference to the opposite categories of 'antagonists' and 'mutualists'; nevertheless, in the absence of indications enabling their circumstantial ascription to one or the other, endophytic fungi are often considered as neutral [4]. Studies on host genotype versus symbiotic lifestyle expression revealed that individual isolates of some fungal species could span the symbiotic continuum by expressing either mutualistic or pathogenic lifestyles in different host plants [5,6]. In recent years, the increasing evidence that many fungal pathogens are able to spread endophytically in unrelated plant species has introduced the perspective that they can actually shift between these categories depending on a series of ecological factors [7]. Indeed, recent papers have reported how fungi

colonizing plants can either directly or indirectly interfere with arthropod development [8], particularly, in the case of fungi producing bioactive secondary metabolites, this adaptation could be related to the toxic or phagodeterrent effects on pests possibly induced by these products [9,10].

The secondary metabolites are not essential for the primary metabolic processes but modulate the microorganism interactions with the surrounding environment [11], underline survival functions by modulating competition, parasitism or symbiosis [12]. These natural compounds also exhibit several biological activities, which may offer potential applications in medicine [13,14] and in agriculture as natural biopesticides [15,16].

Mainly described as a secondary metabolite of *Diplodia* species (Dothideomycetes, Botryosphaeriaceae) [17,18], sphaeropsidin A (SphA, Figure 1) is a pimarane diterpene which was previously reported with the number LL-S491β as a product of a strain of *Aspergillus chevalieri* [19]. However, it is produced also by other fungi which are associated as endophytes with plants [20–26]. This compound has displayed larvicidal and phagodeterrent effects against the yellow fever mosquito (*Aedes aegypti*) (Diptera: Culicidae) [27]. Here we have further explored the spectrum of activity of this compound focusing on a herbivore insect, the lepidopteran *Spodoptera littoralis.*

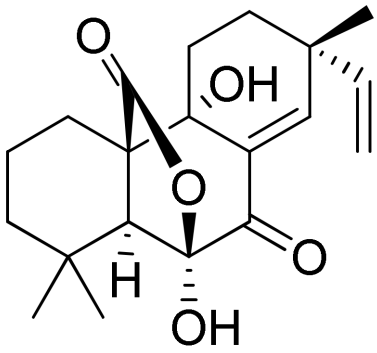

Figure 1. Structure of sphaeropsidin A (SphA).

2. Materials and Methods

2.1. Fungal Strain and Culturing

Diplodia corticola strain (B305) used in this study was previously isolated from *Quercus suber* trees showing canker and dieback symptoms in Algeria. The strain has been identified and characterized, using morphological characters and phylogenetic analysis of molecular data [28]. The nucleotide sequences of B305 are available in GenBank database, under accession numbers MT015626 and MT066136. Liquid cultures of the strain were prepared in Czapek-Dox broth (Oxoid) amended with 2% corn meal in 500 mL Erlenmayer flasks containing 250 mL of the substrate [29] and grown on stationary phase in the dark at 25 °C for 30 days.

2.2. Isolation of SphA from Crude Extract

The culture broth and mycelia were homogenised in a mixer with 350 mL of MeOH (1% NaCl). Subsequently, the suspension was centrifuged for 40 min at 7000 rpm and 10 °C. The pellet was resuspended in 150 mL of a mixture H_2O:MeOH (9:11 *v*/*v*, 1% NaCl) and submitted to a second homogenization followed by centrifugation. Supernatants were collected and MeOH was evaporated under reduced pressure obtaining an aqueous solution for the subsequent extraction (3 times) with ethyl acetate at native pH (=6.0). The organic phases were combined, dried with anhydrous Na_2SO_4 and evaporated under reduced pressure yielding crude extract as brown oil (156.7 mg). The organic extract was purified by column chromatography (CC) on silica gel (40 cm × 1.5 cm i.d.) eluted with $CHCl_3$/*i*-PrOH (19:1, *v*/*v*), originating 8 homogeneous fractions (A: 3.7 mg, B: 6.7 mg, C: 40.3 mg, D: 15.2 mg, E: 9.1 mg, F: 15.9 mg, G: 2.3 mg, H: 32.4 mg), the last of which was

collected by eluting with methanol. Fraction C was purified by TLC on silica gel eluted with *n*-hexane/EtOAc (6:4, *v*/*v*) to obtain SphA (35.4 mg, white crystalline solid, R_f 0.70, in the same chromatographic conditions).

2.3. General Experimental Procedures

Optical rotation of SphA measured in MeOH on a Jasco polarimeter (Tokyo, Japan). ^{1}H NMR spectrum was recorded at 400 MHz in deuterated chloroform ($CDCl_3$) on Bruker (Karlsruhe, Germany) spectrometer and the same solvent was used as internal standards. Thin Layer Chromatography were performed on silica gel plates (Kieselgel 60, F254, 0.25 Merck, Darmstadt, Germany). The spots were visualized by exposure to UV radiation (253 nm), or by spraying first with 10% H_2SO_4 in methanol followed by heating at 110 °C for 10 min. Chromatography was performed on silica gel column (Merck, Kieselgel 60, 0.063–0.200 mm).

2.4. Bioassays on Spodoptera littoralis

Larvae of *S. littoralis* (Lepidoptera, Noctuidae) were reared on artificial diet at 25 ± 1 °C and 70 ± 5% RH, with 16:8 h light-dark period as previously described [30] and used in two different bioassays hereafter described.

2.4.1. Topical Application

Newborn larvae were allowed to grow on the artificial diet until they molted in 2nd and in 5th instar. The 2nd instar larvae were collected and tested in 4 replicates of 25 larvae each (n = 100), while 5th instar larvae (n = 16) were singly treated as described below. The larvae were kept on sterile filter paper in Petri dishes and were directly sprayed with a water/ethanol 50% (*v*/*v*) solution containing SphA at the concentration of 0.4 μg/cm^2, using a fine perfume atomizer. Control larvae were identically treated with a water/ethanol 50% (*v*/*v*) solution (C_{EtOH}) and with water alone (C_{water}). After treatment, the experimental larvae were kept, with a piece of diet (1 cm^2), in 4-well plastic rearing trays (RT32W, Frontier Agricultural Sciences, Pitman, NJ, USA) closed by perforated plastic lids (RTCV4, Frontier Agricultural Sciences). Larval mortality was daily recorded for six days for 2nd instar larvae and until pupation for 5th instar larvae. All bioassays were carried out in duplicate, under the same rearing conditions reported above.

2.4.2. Oral Administration

Newly molted 5th instar larvae, obtained as described above, were anesthetized on ice and 2 μL of a water/ethanol 50% (*v*/*v*) solution, containing SphA at the concentration of 0.02 μg/μL, were poured into the foregut lumen of the larvae by means of a Hamilton Microliter syringe (1701RNR 10 ll, gauge 26 s, length 55 mm, needle 3). Control larvae were treated as described above. The treatment was repeated for 3 consecutive days, for a total amount of 0.12 μg/larvae of SphA. After treatment, larvae were singly isolated in the bioassay tray as described above. Larval development and larval mortality were recorded until pupation: larval weight, pupal weight and the adults' fertility were also recorded. The bioassays were carried out in duplicate, under the same rearing conditions reported above.

2.5. Statistical Analysis

Differences in larval weights were analyzed by One-Way ANOVA followed by the Tukey-Kramer Honestly Significant Difference (HSD) multiple range test ($p < 0.05$). Differences in survival rate were compared by using Kaplan-Meier and long-rank analysis. Data were analyzed using GraphPad Prism version 6.01 (GraphPad software; San Diego, CA, USA).

3. Results and Discussion

SphA (Figure 1) used in this study was obtained as white crystals (35.4 mg) from culture of *D. corticola* B305. In particular, the organic extract was subjected to a chromato-

graphic purification process as described in detail in Section 2.2. This compound was identified on the basis of spectroscopic (^{1}H NMR) and optical rotation data previously determined [13].

Topical application of SphA did not affect the survival rate of both 2nd (Log-Rank test: $p = 0.9437$) (Figure 2A) and 5th instar larvae (100% survival) (Figure 2B). These latter achieved the same weight before pupation (One Way ANOVA. $p = 0.7536$) (Figure 2C) and when they attained the pupal stage (One Way ANOVA: $p = 0.6772$) (Figure 2D).

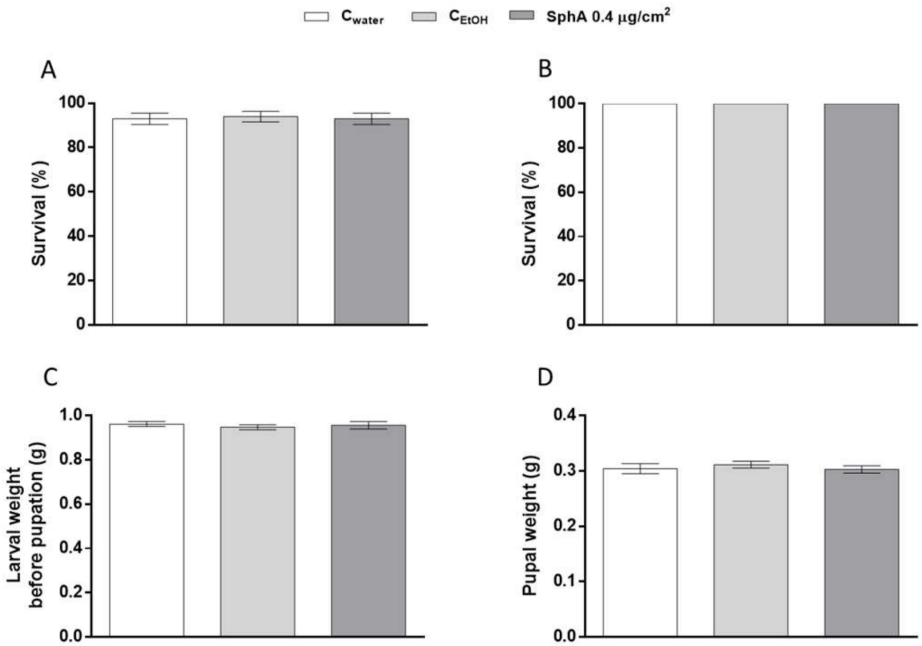

Figure 2. Effect of SphA topical application on *S. littoralis* larvae. SphA sprayed at the concentration of 0.4 µg/cm^2 on *S. littoralis* larvae did not affect the survival rate of 2nd instar (**A**) (Log-Rank test: $\chi^2 = 0.1159$, $p = 0.9437$, dF = 2) and 5th instar larvae (**B**), as well as the larval weight before pupation (**C**) (One Way ANOVA: $F_{(2, 93)} = 0.2838$, $p = 0.7536$) and the pupal weight (**D**) (One Way ANOVA: $F_{(2, 93)} = 0.3914$, $p = 0.6772$). Values are reported as means ± SE.

Experimental larvae orally treated with SphA showed a very strong reduction of the survival rate, which was significantly lower compared to controls (Log-Rank test: $p < 0.0001$) (Figure 3A). The larval mortality started from the last administration of SphA (day 3) and increased over the time until pupation (Figure 3A) with a recorded pupal survival rate of about 67% (for the controls 100%). A significant difference was recorded also for the larval weight before pupation (One Way ANOVA: $p < 0.0001$) (Figure 3B). SphA-treated larvae also showed a modified bodily appearance, and they were smaller than controls (Figure 4). Moreover, despite no alteration of the development time was observed, the pupal weight of the SphA-treated larvae resulted lower than controls (One Way ANOVA: $p < 0.0001$) (Figure 3C). All the adults obtained survived, without differences in their longevity, and no differences in their fecundity was observed (One Way ANOVA: $p = 0.8695$) (Figure 3D).

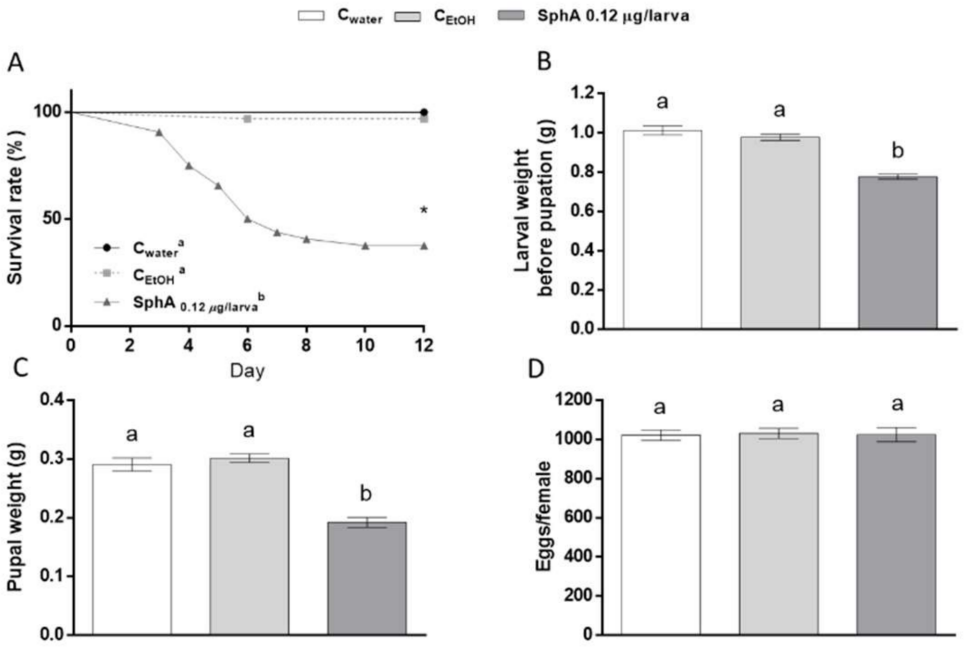

Figure 3. Effect of SphA oral administration 5th instar *S. littoralis* larvae. Orally treated larvae showed a strong reduction of the survival rate compared to controls, decreasing from day three to pupation. (**A**) (Log-Rank test: $\chi^2 = 53.66$, $p < 0.0001$, dF = 2). A significant reduction of the larval weight before pupation (**B**) (One Way ANOVA: $F_{(2, 71)} = 22.14$, $p < 0.0001$) and of the pupal weight (**C**) (One Way ANOVA: $F_{(2, 71)} = 21.41$, $p < 0.0001$) was also observed. No differences were observed in the fecundity of adults obtained from SphA-treated larvae compared with controls (**D**) (One Way ANOVA: $F_{(2, 21)} = 0.1408$, $p = 0.8695$ Asterisk indicate significant differences in the survival curves (Log-Rank test, $p < 0.0001$)). The values in the histograms are means ± SE. Different letters indicate a statistical difference (One Way ANOVA, $p < 0.0001$).

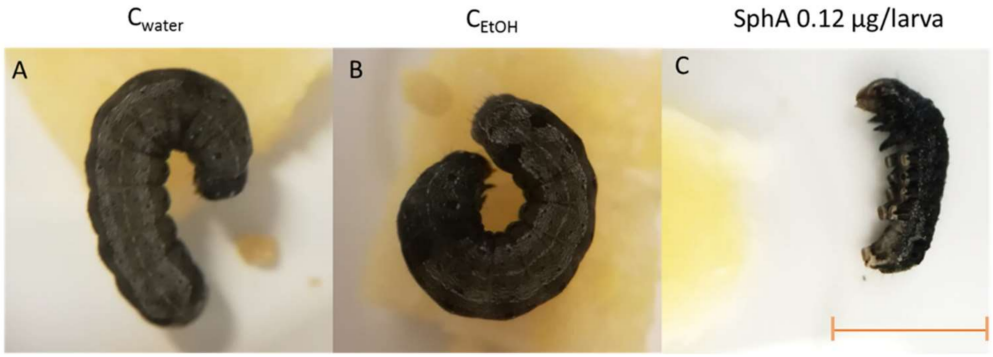

Figure 4. Alteration of *S. littoralis* larval development following oral administration of SphA. Larvae treated with SphA for three days showed a clear reduction of vitality and body size (**C**) No difference in the larval head capsule size indicates that all the larvae are in the same instar. compared to control larvae treated with water (**A**) or EtOH 50% (**B**). Scale bar, 0.5 cm.

Taken together our results indicate that although SphA has no lethal contact activity against *S. littoralis* larvae it showed clear lethal and sublethal effects after ingestion in 5th instar larvae, unequivocally indicating the oral direct toxicity of SphA. Further investigations are needed to better define the insecticidal role of SphA against chewing insects. In case its production by endophytic fungi is demonstrated in planta, SphA might be considered to play a role in the modulation of insect-plant interactions, which is worth of further research efforts aiming to elucidate its mechanism of action and functional role under in vivo conditions.

Author Contributions: A.A. (Anna Andolfi), R.N. and I.D.L. contributed to the study design; G.V., E.R., I.D.L., M.D.G., M.M.S., A.B.-T., A.A. (Artur Alves) and A.E.M., performed the experiments, I.D.L., M.M.S. and A.A. (Anna Andolfi) analyzed the results; I.D.L., A.B. and A.A. (Anna Andolfi) supervised the experiments; R.N., A.A. (Anna Andolfi) and I.D.L. wrote the manuscript. All authors contributed to revise the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. Lu, H.; Wei, T.; Lou, H.; Shu, X.; Chen, Q. A Critical Review on Communication Mechanism within Plant-Endophytic Fungi Interactions to Cope with Biotic and Abiotic Stresses. *J. Fungi* **2021**, *7*, 719. [CrossRef] [PubMed]
2. Zhang, H.W.; Song, Y.C.; Tan, R.X. Biology and chemistry of endophytes. *Nat. Prod. Rep.* **2006**, *23*, 753–771. [CrossRef] [PubMed]
3. Rodriguez, R.J.; White, J.F.; Arnold, A.E.; Redman, R.S. Fungal endophytes: Diversity and functional roles. *N. Phytol.* **2009**, *182*, 314–330. [CrossRef] [PubMed]
4. Sieber, T.N. Endophytic fungi in forest trees: Are they mutualists? *Fung. Biol. Rev.* **2007**, *21*, 75–89. [CrossRef]
5. Redman, R.S.; Dunigan, D.D.; Rodriguez, R.J. Fungal symbiosis: From mutualism to parasitism, who controls the outcome, host or invader? *N. Phytol.* **2001**, *151*, 705–716. [CrossRef]
6. Rodriguez, R.; Redman, R. More than 400 million years of evolution and some plants still can't make it on their own: Plant stress tolerance via fungal symbiosis. *J. Exp. Bot.* **2008**, *59*, 1109–1114. [CrossRef]
7. Salvatore, M.M.; Andolfi, A.; Nicoletti, R. The thin line between pathogenicity and endophytism: The case of *Lasiodiplodia theobromae*. *Agriculture* **2020**, *10*, 488. [CrossRef]
8. Berestetskiy, A.; Hu, Q. The Chemical Ecology Approach to Reveal Fungal Metabolites for Arthropod Pest Management. *Microorganisms* **2021**, *9*, 1379. [CrossRef]
9. Nicoletti, R.; Fiorentino, A. Plant bioactive metabolites and drugs produced by endophytic fungi of Spermatophyta. *Agriculture* **2015**, *5*, 918–970. [CrossRef]
10. Popay, A.J.; Rowan, D.D. Endophytic fungi as mediators of plant-insect interactions. In *Insect-Plant Interactions*, 5th ed.; Bernays, E.A., Ed.; CRC Press: Boca Raton, FL, USA, 2017; pp. 83–104. [CrossRef]
11. Collemare, J.; Navarro-Munoz, J.C.; Mosunova, O. The Biosynthesis of Fungal Secondary Metabolites: From Fundamentals to Biotechnological Applications. In *Encyclopedia of Mycology*, 1st ed.; Zaragoza, O., Casadevall, A., Eds.; Elsevier: Amsterdam, The Netherlands, 2021; pp. 458–476.
12. Demain, A.L.; Fang, A. The Natural Functions of Secondary Metabolites. In *History of Modern Biotechnology I*, 1st ed.; Fiechter, A., Ed.; Springer: Berlin/Heidelberg, Germany, 2000; pp. 1–39.
13. Newman, D.J.; Cragg, G.M. Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. *J. Nat. Prod.* **2020**, *83*, 770–803. [CrossRef]
14. Masi, M.; Maddau, L.; Linaldeddu, B.T.; Scanu, B.; Evidente, A.; Cimmino, A. Bioactive metabolites from pathogenic and endophytic fungi of forest trees. *Curr. Med. Chem.* **2018**, *25*, 208–252. [CrossRef]
15. Cimmino, A.; Masi, M.; Evidente, M.; Superchi, S.; Evidente, A. Fungal phytotoxins with potential herbicidal activity: Chemical and biological characterization. *Nat. Prod. Rep.* **2015**, *32*, 1629–1653. [CrossRef]
16. Marrone, P.G. Pesticidal natural products-status and future potential. *Pest Manag. Sci.* **2019**, *75*, 2325–2340. [CrossRef]
17. Andolfi, A.; Maddau, L.; Basso, S.; Linaldeddu, B.T.; Cimmino, A.; Scanu, B.; Deidda, A.; Tuzi, A.; Evidente, A.A. Diplopimarane, a 20-nor-ent-pimarane produced by the oak pathogen *Diplodia quercivora*. *J. Nat. Prod.* **2014**, *77*, 2352–2360. [CrossRef]
18. Sparapano, L.; Bruno, G.; Fierro, O.; Evidente, A. Studies on structure–activity relationship of sphaeropsidins A–F, phytotoxins produced by *Sphaeropsis sapinea f.* sp. cupressi. *Phytochemistry* **2004**, *65*, 189–198. [CrossRef]
19. Ellestad, G.A.; Kunstmann, M.P.; Mirando, P.; Morton, G.O. Structures of fungal diterpene antibiotics LL-S491. beta. and-. gamma. *J. Am. Chem. Soc.* **1972**, *94*, 6206–6208. [CrossRef]
20. Morooka, N.; Tatsuno, T.; Tsunoda, H.; Kobayashi, K.; Sakurai, T. Chemical and Toxicological Studies of the Phytotoxin, 6α,7β,9α-Trihydroxy-8,15-isopimaradiene-20,6-γ-lactone, Produced by a Parasitic Fungus, *Phomopsis* sp., in Wilting Pine Trees. *Agric. Biol. Chem.* **1986**, *50*, 2003–2007.
21. Weber, R.W.; Kappe, R.; Paululat, T.; Mösker, E.; Anke, H. Anti-Candida metabolites from endophytic fungi. *Phytochemistry* **2007**, *68*, 886–892. [CrossRef]

22. Wang, X.N.; Bashyal, B.P.; Wijeratne, E.K.; U'Ren, J.M.; Liu, M.X.; Gunatilaka, M.K.; Arnold, A.E.; Gunatilaka, A.L. Smardaesidins A–G, Isopimarane and 20-nor-Isopimarane Diterpenoids from *Smardaea* sp., a Fungal Endophyte of the Moss Ceratodon purpureus. *J. Nat. Prod.* **2011**, *74*, 2052–2061. [CrossRef]
23. Yan, T.; Guo, Z.K.; Jiang, R.; Wei, W.; Wang, T.; Guo, Y.; Song, Y.C.; Jiao, R.H.; Tan, R.X.; Ge, H.M. New flavonol and diterpenoids from the endophytic fungus *Aspergillus* sp. YXf3. *Planta Med.* **2013**, *79*, 348–352. [CrossRef]
24. Li, Y.; Scott, R.; Hooper, A.R.; Bartholomeusz, G.A.; Kornienko, A.; Bills, G.F. *Aspergillus candidus* is a newly recognized source of sphaeropsidin A: Isolation, semi-synthetic derivatization and anticancer evaluation. *Bioorg. Med. Chem. Lett.* **2017**, *27*, 5436–5440. [CrossRef] [PubMed]
25. Neuhaus, G.F.; Adpressa, D.A.; Bruhn, T.; Loesgen, S. Polyketides from marine-derived *Aspergillus porosus*: Challenges and opportunities for determining absolute configuration. *J. Nat. Prod.* **2019**, *82*, 2780–2789. [CrossRef] [PubMed]
26. Yang, H.; Liu, X.Y.; Zhang, P.L.; Gao, H.M.; Zhang, L.T.; Shen, T.; Ren, D.M.; Lou, H.X.; Wang, X.N. New terpenoids and triketides from culture of the fungus *Botrysphaeria laricina*. *Fitoterapia* **2020**, *147*, 104758. [CrossRef] [PubMed]
27. Cimmino, A.; Andolfi, A.; Avolio, F.; Ali, A.; Tabanca, N.; Khan, I.A.; Evidente, A. Cyclopaldic Acid, Seiridin, and Sphaeropsidin A as Fungal Phytotoxins, and Larvicidal and Biting Deterrents against *Aedes aegypti* (Diptera: Culicidae): Structure-Activity Relationships. *Chem. Biodivers.* **2013**, *10*, 1239–1251. [CrossRef]
28. Mahamedi, A.E.; Phillips, A.J.; Lopes, A.; Djellid, Y.; Arkam, M.; Eichmeier, A.; Zitouni, A.; Alves, A.; Berraf-Tebbal, A. Diversity, distribution and host association of Botryosphaeriaceae species causing oak decline across different forest ecosystems in Algeria. *Eur. J. Plant Pathol.* **2020**, *158*, 745–765. [CrossRef]
29. Salvatore, M.M.; DellaGreca, M.; Nicoletti, R.; Salvatore, F.; Tuzi, A.; De Tommaso, G.; Alves, A.; Mahamedi, A.E.; Berraf-Tebbal, A.; Andolfi, A. Mitidjospirone, a new spirodioxynaphthalene and GC-MS screening of secondary metabolites produced by strains of Lasiodiplodia mitidjana associated to Citrus sinensis dieback. *Nat. Prod. Res.* **2021**, 1–10. [CrossRef]
30. Di Lelio, I.; Varricchio, P.; Di Prisco, G.; Marinelli, A.; Lasco, V.; Caccia, S.; Casartelli, M.; Giordana, B.; Rao, R.; Gigliotti, S.; et al. Functional analysis of an immune gene of *Spodoptera littoralis* by RNAi. *J. Insect Physiol.* **2014**, *64*, 90–97. [CrossRef]

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Biological and molecular characterization of *Aromia bungii* (Faldermann, 1835) (Coleoptera: Cerambycidae), an emerging pest of stone fruits in Europe

Elia Russo[1], Francesco Nugnes[1]✉, Francesco Vicinanza[2], Antonio P. Garonna[2] & Umberto Bernardo[1]

The red-necked longhorn beetle (RLB) *Aromia bungii* (Fald.) is an emerging pest of stone fruit trees, native to East Asia, accidentally introduced in Europe (Germany and Italy) and Japan. Threatening seriously the stone fruit crops in Europe, RLB was added to both the EPPO A1 and priority pest lists of quarantine species. Molecular analyses highlighted that all specimens recovered in southern Italy share the same haplotype, different from the German one, supporting that the invasive process in Europe started from at least two independent introductions. To fill the existing gap of biological knowledge about *A. bungii*, several laboratory tests were carried out on specimens collected in the outbreak area of Naples (Italy). Results suggest a high biotic potential of the RLB Italian population. Females showed a short pre-oviposition period while the period of oviposition lasted about three weeks, with a rate of 24.2 eggs/day. Each female laid an average of 587.5 eggs and spawned the largest amount of eggs during the first week after emergence. Fed males live up to 62 days at 20 °C while fed females about 63 days at 25 °C. These results are crucial to draw up a multi-facet IPM approach against *A. bungii* in the outbreak areas.

The growing international trade of living plants and plant-based products increases the likelihood of introducing alien organisms to new areas, where they may develop as invasive pests. Italy is particularly troubled by this phenomenon because of its geographical position and habitat patterns favourable to the spread and the establishment of allochthonous insect species[1], with dramatic economic and ecological impacts to agricultural and natural forested areas[2–4].

The red-necked longhorn beetle (RLB) *Aromia bungii* (Faldermann, 1835) (Coleoptera: Cerambycidae) is an invasive wood-borer pest of *Prunus* trees, including important commercial varieties. *Aromia bungii* belongs to the subfamily Cerambycinae, tribes Callichromatini, and it is native to Eastern Asia (China, Korea, Taiwan, Vietnam and Mongolia)[5,6], where it is considered the major pest of stone fruit trees such as peach, apricot, plum and cherry[5,7].

The first field records of this pest outside its native area date back to 2011, when RLB was found in Bavaria (Germany) on *Prunus domestica* subsp. *insititia*[8]. A year later, as a result of an important outbreak found in Southern Italy, *A. bungii* was added to the EPPO Alert List[9]. Its presence was officially reported after the record of massive infestations that affected different host species (*P. armeniaca*, *P. domestica*, and *P. avium*), both in commercial orchards and in private gardens located in Naples and in a few neighbouring municipalities. In 2013, a new outbreak was recorded in Northern Italy near Milan[10] leading to the inclusion of RLB in the A1 list of quarantine pests[11]. Because the presence of *A. bungii* is known in a limited area of the European Union and due to its more serious economic, environmental, and social impacts than other harmful quarantine organisms, it has been recently included in the priority list of relevant quarantine pests to the Union territory[12].

Aromia bungii is currently established in an area of about 250 km^2 in the Campania Region and is the focus of an eradication effort there[13]. The pest is in a containment state in Northern Italy[14,15], while in Germany it is

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Sex [test]	Body length	Elytra	Metatibia
Male (n = 60)	28.4 ± 0.42 (17-36)	17.4 ± 0.28 (9-22.5)	10.5 ± 0.15 (6–13)
[Longevity]			
Female (n = 60)	31.5 ± 0.46 (20–40)	19.9 ± 0.27 (13–25)	11.3 ± 0.17 (8–15)
[Longevity]			
Female (n = 10)	29.8 ± 0.49 (27–31)	20.5 ± 0.40 (18.5–22)	10.9 ± 0.35 (9–12)
[Dissected]			
Female (n = 18)	30.3 ± 0.54 (24–35)	18.7 ± 0.44 (13–21)	10.4 ± 0.26 (9–13)
[Fecundity]			

Table 1. Size of *A. bungii* adults in mm (mean ± SE) with the range of variation in round brackets. In square brackets is the type of experiments that measured specimens were used for.

Sample	Localities	Province	Host	Year	Coordinates	Genbank Accession Code	
						COI	28S-D2
ABA1	Riserva Astroni	Naples	*P. armeniaca*	2013	40°50′N, 14°09′E	MN662926	MN658539
ABA2						MN662927	MN658540
ABA3	Via Cinthia		*P. domestica*	2013	40°50′N, 14°11′E	MN662928	MN658541
ABA4						MN662929	MN658542
ABA5	Via Cavone		*P. avium*	2017	40°52′N, 14°13′E	MN662930	MN658543
ABA6						MN662931	MN658544
ABA7			*P. domestica*	2017		MN662932	MN658545
ABA8						MN662933	MN658546
ABA9	Via Campana		*P. armeniaca*	2017	40°50′N, 14°07′E	MN662934	MN658547
ABA10						MN662935	MN820636

Table 2. Localities and host species where RLB stages were collected. For each specimens COI and 28S-D2 were sequenced.

considered as transient, under eradication[16,17]. *Aromia bungii* has also been reported in Japan[6,18] where it appears to be spreading rapidly, threatening the cherry blossom trees across the country[6,19,20]. Wood packaging material or wooden products infested by immatures might be behind the accidental introduction of RLB in Europe and Japan[21] as reported for other invasive longhorn beetles[22,23].

Although most longhorn beetles develop on dead or decaying trees, *A. bungii* develops on healthy host plants, infesting the trunks and main branches[5,24]. Infested trees may be easily identified by the presence of abundant frass at the tree base and exit holes on the bark[5,24–27]. The life cycle lasts 1–4 years showing a clear latitudinal gradient[5,25,28].

As a result of the new pest status of RLB, recent research has shed some light on morphological structures, flight behaviour, semiochemicals, intraspecific communication, mate location, and natural control[29–34]. In China, authors investigated mainly *A. bungii* distribution, life cycle, bionomics, injury levels, and control options[25,26,28,35]. Nevertheless, its biology and genetic variability are not well known, and some reproductive traits, such as lifetime fecundity, are unexplored[21]. Knowledge of these parameters is essential to assess the potential damage of the invasive pest, to develop methods of survey and monitoring, and the screening of management options of RLB in the invaded countries. Our objectives were to genetically characterize the *A. bungii* population in southern Italy, and evaluate its reproductive patterns and biological traits under laboratory conditions.

Results

Collection of RLB adults from infested logs. A total of 310 adult individuals of *A. bungii* emerged in entomological cages (156 females and 154 males, sex ratio 0.5) at 25 ± 1 °C. The first adults emerged on 24 April and emergence continued until 9 July (77 days). All specimens used in the tests were measured and results are summarized in Table 1. Body length was chosen as an indicator of body size to verify correlations with biological variables, because it was related to metatibia ($r = 0.947$, $F = 2783.03$; $df = 1, 156$; $P < 0.00001$) and elytral length ($r = 0.971$, $F = 5201.49$; $df = 1, 156$; $P < 0.00001$).

Morphological and molecular identification. COI sequencing revealed that all the *A. bungii* samples collected in Campania shared the same mitochondrial haplotype (Table 2). Blast search showed that the haplotype found in Campania had five and two mismatches with sequences from China (KF737790) and Germany (KM443233), respectively. The other sequence of *A. bungii* from China available in GenBank (DQ223728) overlapped only for 397 out of 658 bp with our haplotype, with two mismatches. Genetic distance between the two Chinese sequences was 1.07% (±0.005). The highest inter-group distance among *A. bungii* COI haplotypes resulted between Chinese sequences and Campanian ones (0.7%), while the lowest between German and Campanian haplotypes (0.4%).

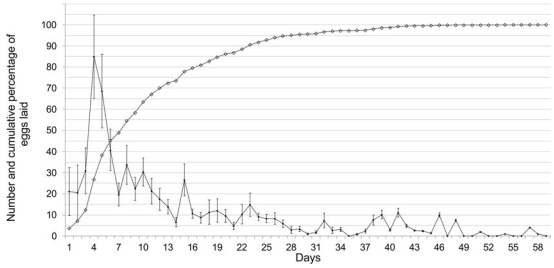

Figure 1. Daily distribution (mean ± SE) and cumulative percentage of eggs laid at 25 °C by *A. bungii* females.

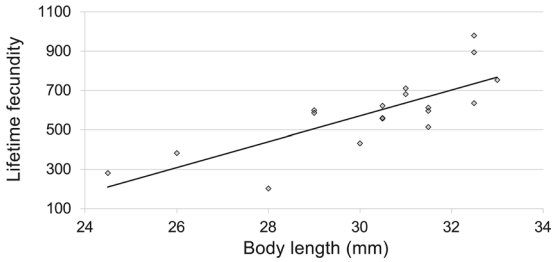

Figure 2. Regression between lifetime fecundity and body length in *A. bungii* ovipositing females (n = 18); Lifetime fecundity = −1392.85 + 65.4061*Body length; F = 24.91; df = 1, 16; $P = 0.0001$.

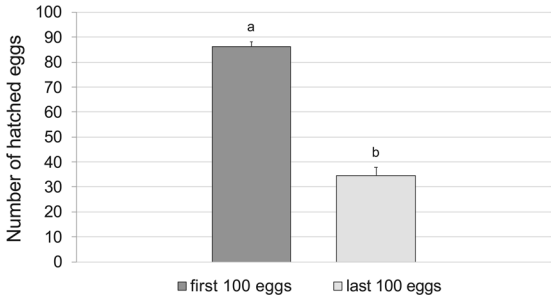

Figure 3. Comparison between the number of hatched eggs (mean + SE) of the first and the last 100 eggs laid by *A. bungii* (n = 18). Bars with different letters indicate significant difference at the 5% confidence level.

Aromia bungii samples collected in Campania also shared the 28S-D2 haplotype, which is identical to the only two present in GenBank (accession numbers HQ832606 and KF142125) whose origins are presumably Chinese.

Fecundity tests. Females started oviposition 2.1 ± 0.18 days after their emergence (pre-oviposition period) and proceeded until the 58th day. The mean OP was 22.1 ± 2.96 days. Daily distribution showed that the highest number of eggs laid per day was 84.8 ± 20 and was recorded on the 4th day since emergence (Fig. 1). The mean LF was 587.5 ± 44.97 eggs and females laid 50 ± 4% of eggs in the first week after emergence, with the number of eggs laid per day decreased gradually in the following weeks (Fig. 1). Mean OR was 24.2 ± 3.51 eggs per day. Mean longevity of ovipositing females was 34.7 ± 2.74 days, and a weak relationship with LF was found (r = 0.195, F = 5.11; df = 1, 16; $P = 0.038$). Multiple regression analysis reported the following standardized regression equation: $\log_{10}$ LF = 0.0358 $\log_{10}$ OP + 0.0370 $\log_{10}$ OR. This indicates that OP and OR had almost the same effect on the LF. A moderately strong relationship was found between LF and female body length (Lifetime fecundity = −1392.85 + 65.4061*Body length; F = 24.91; df = 1, 16; $P = 0.0001$; r = 0.5844) (Fig. 2), while a relatively weak correlation (r = 0.192, F = 5.04; df = 1, 16; $P = 0.039$) resulted between female body length and longevity.

Fertility and hatching period. The hatching rate was 75.6 ± 2.30% (range: 58.7–86.7%). Fertility was not correlated with body length (r = −0.058, F = 0.06, df = 1, 16; $P = 0.81$), longevity (r = −0.010, F = 0.84; df = 1, 16; $P = 0.374$) or LF (r = −0.053; F = 0.14; df = 1, 16; $P = 0.71$). Differences in hatching rate between the first and last 100 eggs laid showed that the earlier eggs were more fertile than the later ones (ANOVA, F = 312.27; df = 1, 34; $P < 0.0001$) (Fig. 3). Eggs hatched 8.7 ± 0.03 days after laying (range: 7–11 days). The highest number of eggs (38.6 ± 7.62%) hatched on the 8th day of oviposition (Fig. 4).

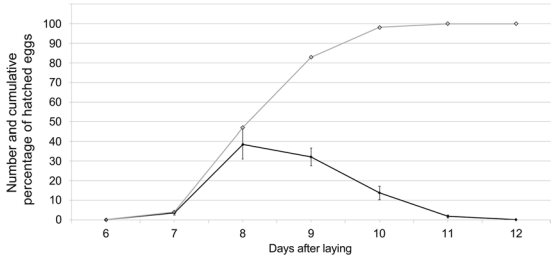

Figure 4. Hatching period (days after laying) and cumulative percentage of hatched eggs of *A. bungii* (mean + SE) at 25 °C (first 100 laid eggs).

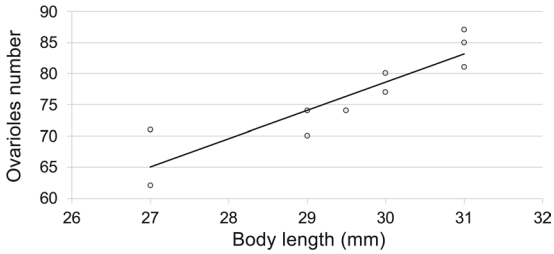

Figure 5. Regression between ovarioles and body length in newly emerged *A. bungii* females (n = 10); Ovarioles number = −57.2077 + 4.52658*Body length; F = 35.09; df = 1, 8; *P* = 0.0004.

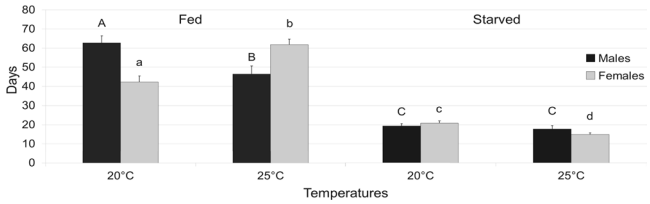

Figure 6. Longevity of fed (left) and starved (right) adults (♂ and ♀) of *A. bungii* (mean + SE) at two different temperatures (bars with different upper and lower case letter indicate significant difference at 5% confidence level).

Ovarioles number and ovigeny index. The mean number of ovarioles per ovary was 76.1 ± 2.38 (n = 10) and ranged between 62 and 85. A relatively strong relationship was found between ovarioles number and female length (Ovarioles number = −57.2077 + 4.52658*Body length; F = 35.09; df = 1, 8; *P* = 0.0004; r = 0.7911) (Fig. 5). Newly eclosed females had a mean of 385.6 ± 22.74 mature eggs. The ratio between egg load at emergence and realized fecundity indicated that *A. bungii* is a slightly pro-ovigenic species. Ovigeny index was 0.66 ± 0.003.

Adult longevity. Among adults that were provided with food, males lived significantly longer at 20 ± 1 °C (62.7 ± 3.75 days) than at 25 °C (ANOVA, F = 8.18; df = 1, 28; *P* = 0.008), while the longest non ovipositing female lifespan was recorded at 25 ± 1 °C (61.9 ± 2.81 days, n = 15) (ANOVA, F = 21.4; df = 1, 28; *P* = 0.0001) (Fig. 6). The latter lived significantly longer than ovipositing females (34.7 ± 2.74 days, n = 18) (ANOVA, F = 47.3; df = 1, 31; *P* < 0.0001) (Fig. 6). Starved females (20.9 ± 1.25 days) lived significantly longer at 20 ± 1 °C, than at 25 °C (ANOVA F = 15.51; df = 1, 28; *P* = 0.0005), while no difference was found between starved males at the two tested temperatures (ANOVA, F = 0.51; df = 1, 28; *P* = 0.48) (Fig. 6). A moderately strong relationship was found between body length and longevity for fed females at 25 ± 1 °C [r = 28.5, F = 6.6; df = 1, 13; *P* = 0.02], while no relationship was found for males [r = −0.004, F = 0.94; df = 1, 13; *P* = 0.35].

Discussion

Multiple or single introduction of an invasive species can be discriminated by the identification of the haplotypes involved in the invasion process[36–38]. Molecular analysis of the Italian (southern Italy) specimens showed no haplotype variability; this could be due to the founder effect (reduced or no genetic variation that occurs when a population is established by a single or a few specimens)[39]. This suggests that *A. bungii* arrived in Italy by a single introduction event and with few individuals. This is a very common pattern found also in other invasive species recently recorded in Italy[40,41].

The genetic variability of the *A. bungii* populations in the area of origin is not yet well defined, and the single haplotype found in Campania did not match either the only haplotype known from China nor the only haplotype found in Germany. Hence, to date we are not able to establish the region of origin of studied specimens, but we

can speculate that the invasive process in Europe started from at least two independent introductions occurred in continental and peninsular Europe, respectively.

Knowledge of biological traits of invasive species is strategic to design the best control strategies. Colonisation and damage levels depend on several factors, especially on the reproductive potential of the invaders[42]. Sex ratio of *A. bungii* obtained in this study is slightly higher than the values reported by other authors (0.5 vs 0.43)[26]. All adults used in the present study were obtained from infested material collected in the field. In accordance with previous studies[5,43], the mean adult body length was 31.2 and 29.2 mm for females (n = 93) and males (n = 65), respectively. Our estimate of mean lifetime fecundity of *A. bungii* is 587.5 eggs (ranging from 201 to 978), which is within the ranges reported in the Cerambycinae subfamily[44,45]. Previous observations carried out in China showed that RLB females can lay in average 324.6 to 357.0 eggs (ranging from 91 to 734), but the authors did not specify the number of replicates and pairs managements[26,35]. Pair management is very important and can strongly affect the number of laid eggs, because if adults are not regularly coupled, oviposition is reduced, as already reported for other species of cerambycids[46,47]. Investigations on LF in the genus *Aromia* are limited to *A. moschata* (Linneus 1758), the musk beetle. Duffy[48] reported for this congeneric species an average number of eggs laid much lower than that recorded for RLB in this study. This is certainly due to the different size of the eggs belonging to the two species as compared by us. The volume of the egg of *A. moschata* is about five times that the one of *A. bungii* (Garonna, personal observation). Our estimates of *A. bungii* lifetime fecundity are also higher than those known for other wood-borer pests in the same subfamily, such as *Trirachys sartus* (Solsky, 1871) and *Osphranteria coerulescens* Redtenbacher, 1850[49,50]. Path analysis indicated that female body size expressed by body length affected positively the LF. A positive correlation between LF and female size is very common in longhorn beetles belonging to both Cerambycinae and Lamiinae[51].

The number of RLB ovarioles per female was variable, ranging from 62 to 85. A positive correlation with body length indicated that long-sized females have a greater ovarian mass and egg productivity per ovariole compared with small females. Ovigeny index (0.66) showed that *A. bungii* is a slightly pro-ovigenic species, meaning that the female emerges with just over half of her eggs mature. This result is congruent with the record that some females laid the first eggs within 24 hours after emergence, with the short pre-oviposition period (2.1 days), and with the high number of eggs laid in the first week. So far, most of the longhorn beetles studied have been reported as synovigenic species, but the ovigeny pattern has been calculated through a different methodology (daily fecundity, pre-oviposition and oviposition period)[51–54].

The oviposition period of *A. bungii* was highly variable among tested females ranging from 18 to 58 days and the mean value was about a month, similar to 26.3 days reported by Hu *et al.*[26] and comparable to that of other longhorn beetles like the lamiine *Monochamus urussovii* (Fischer - Waldheim, 1806) and *Paraglenea fortunei* (Saunders, 1853)[53,55]. Conversely, OP was lower than that of cerambycine *Cerambyx welensii* Küster, 1846 and *Cerambyx cerdo* Linneus, 1758[51,54]. Mean OR averaged 22.1 eggs and was very close to the reported value in the subfamily Cerambycinae[51]. The highest mean number of laid eggs per day in *A. bungii* was 84.8 and the considerable oscillations during females LF, with inconsistent values ranging from 0 to 281 eggs per day, are common in longhorn beetles[51,54,56] and often related to intraspecific differences in female size, oogenesis rate or ovarioles number[57]. Rate and period of oviposition had a similar effect on LF of *A. bungii*, differently from aforementioned lamiine species where the oviposition period contributed almost twice to the difference in lifetime fecundity than the rate of oviposition[53,55].

About 75% of RLB eggs hatched and the mean egg developmental time was 8.7 days, at 25 °C. Previous laboratory studies on *A. bungii* have reported higher fertility values ranging between 94.3 and 97.6% and congruent values of egg developmental time (4 to 14 days)[26]. However, in the latter study, no data about the laboratory conditions of the studies, particularly temperatures, were provided. Abiotic factors, such as temperature, are known to be particularly crucial in affecting insect life history such as developmental period, longevity, fecundity, and fertility, as well as flight and behaviour[58]. In coleopteran species and, in particular, in longhorn beetles, it is widely demonstrated that temperatures strongly affect embryonic development and thus the egg developmental times[59–62]. Tests conducted at similar temperatures on the invasive species *Anoplophora glabripennis* (Motschulsky, 1853), showed lower fertility[61] than those observed for *A. bungii* in the present study, while egg developmental time of RLB was similar to the cerambycine *Xylotrechus arvicola* Olivier, 1795[62]. The earlier eggs laid by RLB were more than twice as fertile as the later ones. Offspring rate production, indeed, is variable during insect lifetime; egg fertility decreases towards the end of the female lifespan[58,63,64].

Fed females of *A. bungii* lived on average two months and about two weeks longer than males, at 25 °C. Longevity recorded in the present study is congruent with that of previous laboratory studies on cerambycids in which females usually lived longer than males and the mean adult longevity values ranged from about one month to more than seven months[65]. Conversely, at 20 °C, fed males lived longer than fed females, as reported also for the cerambycine *Phoracantha semipunctata* (Fabricius, 1775) and the lamiine *An. glabripennis*[61,66]. Not surprisingly, starved RLB adults lived two weeks less than fed at both tested temperatures, unlike the starved adults of the lamiine *Glenea cantor* (Fabricius, 1787) that died only a few days after emergence (2.47 and 4.71 days for males and females, respectively)[52]. In longhorn beetles, it is widely demonstrated that fed adults are more long-lived than starved[47]. Laying females used in fecundity tests showed a shorter lifespan than virgin females (about half), at 25 °C. This result confirms that the longevity of female decrease when individuals invest in the cost of reproduction, mating in particular[67,68].

Information on the reproductive biology of exotic species is one of the requirements for successful eradication programs against invasive pests[42]. The data collected in this study suggests that a successful control strategy of RLB is hard to implement, and eradication measures need that all infested trees must be cut, chipped, and set on fire. Simultaneously, an early and correct flight monitoring of RLB adults is necessary for determining the best strategy to improve its management. Recent research on the efficacy of the identified pheromones of RLB[31,33,34] has opened interesting monitoring and control scenarios (i.e., mass trapping) where outbreaks of the pests are

recorded. Timing of insecticide treatments is another critical issue. Currently, a single active ingredient is registered in Italy for crop use against *A. bungii* (deltamethrin with three applications per year)[13]. This product may not be enough effective in killing emerging adults because of protracted emergence period and extended adult longevity. In addition, the short pre-oviposition period reduces the effectiveness of this control method. However, in an Integrated Pest Management (IPM) approach, additional practices must be combined, including physical, cultural and biological strategies. Experimental methods of biological control by entomopathogenic nematodes and fungi, already studied for other invasive wood-borer pests as *An. glabripennis* and *Anoplophora chinensis* (Forster, 1771)[7,22], could be applied as containment measures to RLB to significantly reduce its biotic potential.

Conclusion

This is the first detailed study on molecular and biological characterization of the invasive pest *A. bungii*, focusing on the basic aspects of the reproductive biology, in particular on its lifetime fecundity. All the specimens collected in Italy share the same mitochondrial haplotype, suggesting a single introduction or multiple introductions from the same source. Biological traits obtained show that RLB has a high biotic potential that can allow the beetle to establish, in a short time, a harmful population in a new area, if it will be accidentally introduced, as it happened in southern Italy and Japan. Biological data here presented will be useful to establish new action guidelines to contain the infestations of *A. bungii* in the outbreak areas, and to carry out an adequate IPM program in southern Italy. However, further in-depth studies are needed, in particular to clarify the duration of its still largely unknown life cycle.

Methods

Collection of RLB from infested logs. Adults and immature stages of *A. bungii* were obtained from infested plants in the outbreak area west of Naples (Campania Region, southern Italy, 40°50.423′N, 14°10.862′E and 40°50.340′N, 14°10.767′E) that were cut down during winter 2016–2017 under the eradication programme coordinated by the Regional Plant Health Service. The area included non-specialized orchards where stone fruits are predominant (especially *Prunus domestica*, *P. armeniaca*, and *P. avium*).

RLB infested trees were recognized by the presence of frass at tree base and along branches. The presence of typical exit holes and mature larvae during trunks inspection allowed the recovery/collection of samples to employ in the scheduled biological studies. Infested plants were cut and divided into 50–60 cm logs, sealed at both ends with a double layer of polyester nonwoven and moved to the laboratory. Trunks were stored in entomological cages and placed in a quarantine facility at 25 ± 1 °C, 60 ± 5% relative humidity (RH) and photoperiod 16 L:8D to allow adult emergence.

Emerged adults were daily collected and individually stored in plastic containers to prevent adults from injuring each other and randomly assigned to the different tests. For each adult, body length (from mandibles to anal pore along the ventral surface), elytra, and metatibia were measured. Few deformed or dying adults were discarded. Sex ratio was calculated following Kenneth and Hardy[69]. At the end of the tests, all wooden material was burned. Any adult beetles and larvae that remained alive were frozen at −20 °C.

Morphological and molecular identification of RLB. Adults and mature larvae of *A. bungii* were identified following the taxonomic keys, descriptions and comparative images available in Matsushita[70], Gressit[5] and Duffy[24]. To characterize *A. bungii* and to estimate the genetic diversity of introduced populations, several samples were collected on different recorded host species in four localities (Table 2).

Aromia bungii DNA was extracted from single individuals listed in Table 2 through a slight modification of Chelex and proteinase K based method described in Gebiola *et al.*[71]. For each sample, a foreleg with small shreds of muscular tissue was separated with tweezers, soaked in a solution of 6 µl proteinase K (20 mg/ml) and 100 µl (5%) Chelex 100 (Bio-Rad, Richmond, CA) and incubated at 55 °C for 2 hours.

Extracted DNA was employed to amplify both a portion of the mitochondrial gene *Cytochrome c Oxidase* subunit I (COI) and the ribosomal gene 28S-D2 using primer pairs LCO/HCO[72] and D2F/D2R[73], respectively. PCR reactions and cycling conditions for COI and 28S-D2 were set as described in Gebiola *et al.*[71].

PCR products were checked on a 1.2% agarose gel stained with GelRED (Biotium, Fremont, CA, USA) and sequenced. Chromatograms were assembled and edited by eye with Bioedit 7.2.5[74]. Edited COI sequences were virtually translated into the corresponding amino acid chain to detect frame-shift mutations and stop codons, using EMBOSS Transeq [http://www.ebi.ac.uk/Tools/st/emboss_transeq/(accessed 10 sep 2019)]. Edited sequences were checked against the GenBank database by Blast nucleotide searches and were submitted to the GenBank database with accession numbers as in Table 2. COI haplotypes distances and standard errors were calculated with MEGA 6 software[75] as "uncorrected *p*-distance" including homologous sequences of RLB available in GenBank (accessed 13 Sep 2019).

Fecundity tests. RLB lifetime fecundity (LF) was assessed by using 18 females. Fresh and uninfested *P. armeniaca* branches with regular bark and free from protruding twigs were selected to obtain logs with a mean length of 35 cm and a mean diameter of 7 cm. Newly emerged females were allowed to mate with a male for about two hours and then placed in a transparent plastic container (45 × 30 × 25 cm) covered by a rigid tulle fabric. Each female was daily fed with a fresh apple piece and a new log was placed in the container to allow oviposition until female death. Weekly, a male was placed for 24 h in the same container for mating. Every 24 h all logs were examined under a binocular microscope at X 40 magnification to count the eggs laid and then kept at 25 ± 1 °C until egg hatching. The longevity of ovipositing females was recorded. We determined the following variables for each female: 1) lifetime fecundity (LF = total number of eggs laid during her lifetime); 2) oviposition period (OP = the time elapsed between the first and last egg laid); and 3) oviposition rate (OR = mean number of eggs

laid per day during the OP, i.e., OR = LF/OP) as proposed by Togashi and Yamashita[53]. Correlations between body size, longevity, and LF of ovipositing females were assessed.

Fertility and hatching period. Fertility was calculated as the hatching rate of laid eggs by single female used in the fecundity tests[58]. Logs exposed 24 h to RLB adults were observed daily. Hatched eggs were labeled each day with a different color and hatching rate was recorded. Eggs were considered hatched when newborn larvae penetrated the log bark and light frass was produced around the oviposition site. The fertility of the first and last 100 eggs laid by each female were compared. The hatching period (i.e., days after deposition) was assessed on the first 100 eggs laid by each female.

Ovarioles number and ovigeny index. Numbers of ovarioles and full size eggs per female were estimated by dissecting females within 24h after emergence in 0.8% saline solution (n = 10). The ovigeny index was calculated by dividing the initial mean load of mature egg by the maximum average lifetime fecundity (i.e., realized fecundity *sensu*)[76].

Adult longevity. Adult longevity was evaluated on fed and starved adults (n = 15) at two different temperatures (20 and 25 ± 1 °C) with photoperiod 16 L:8D and RH of 60 ± 5%. Newly emerged adults were singly isolated in polyethylene bottle (bottom diameter 67, top diameter 50 and height 90 mm with a tulle cover) and assigned to one of the temperature treatments. Adults were daily provided with apple pieces as fresh food and checked for mortality. After death, elytra, metatibia, and body length were measured on each tested adult. The possible correlation between longevity and body size was evaluated only on fed males and females at 25 ± 1 °C. To evaluate the effect of oviposition on lifespan, the longevity of fed females at 25 ± 1 °C was compared with that of fed females used in the fecundity test.

Statistical analysis. Data satisfying conditions of normality and homoscedasticity, both untransformed or after appropriate transformation (cosine transformed, in the comparison between the first 100 and the last 100 eggs laid), were analysed by ANOVA and the means were separated at the 0.05 level of significance by a multiple range test (Tukey HSD or, in case of unequal samples, Bonferroni)[77]. All relationships between variables were assessed by regression analysis. To determine the effects of OP and OR on differences in lifetime fecundity among females, a multiple regression analysis was performed to obtain the standardized partial regression coefficients of $\log_{10}$ OP and $\log_{10}$ OR. All data are presented non-transformed, with standard error within brackets.

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References

1. Lupi, D. *et al.* Insects and globalization: Sustainable control of exotic species in Italian agro-forestry ecosystems. *Landsc. Manag. Funct. Biodivers. IOBC-WPRS Bull.* **100**, 87–90 (2014).
2. Bosco, L., Moraglio, S. T. & Tavella, L. Halyomorpha halys, a serious threat for hazelnut in newly invaded areas. *J. Pest. Sci.* **91**, 661–670, https://doi.org/10.1007/s10340-017-0937-x (2018).
3. Garonna, A. P. *et al.* The spread of the non-native pine tortoise scale Toumeyella parvicornis (Hemiptera: Coccidae) in Europe: a major threat to Pinus pinea in Southern Italy. *iForest* **11**, 628–634, https://doi.org/10.3832/ifor2864-011 (2018).
4. Nugnes, F., Russo, E., Viggiani, G. & Bernardo, U. First record of an invasive fruit fly belonging to Bactrocera dorsalis complex (Diptera: Tephritidae) in Europe. *Insects* **9**, 182, https://doi.org/10.3390/insects9040182 (2018).
5. Gressitt, J. L. Destructive long-horned beetle borers at Canton, China. *Spec. Publ. Lingnan. N. H. Surv. Mus.* **1**, 1–60 (1942).
6. Kano, M., Nonaka, T., Kiriyama, S. & Iwata, R. Aromia bungii (Coleoptera: Cerambycidae), an invasive cerambycid, found at Soka, Saitama Pref., Japan, infesting cherry trees, Cerasus × yedoensis 'Somei-yoshino'. *For. Pests* **63**, 101–105 (2014).
7. Hu, J., Angeli, S., Schuetz, S., Luo, Y. & Hajek, A. E. Ecology and management of exotic and endemic Asian longhorned beetle Anoplophora glabripennis. *Agric. For. Entomol.* **11**, 359–75 (2009).
8. Burmeister, E. G., Hendrich, L. & Balke, M. Der asiatische moschusbock Aromia bungii (Faldermann, 1835) - Erstfund für Deutschland (Coleoptera: Cerambycidae). *Nachr. Bl. Bay. Ent.* **61**(1-2), 29–31 (2012).
9. EPPO First report of Aromia bungii in Italy. EPPO Reporting Service, 2012/204 (2012).
10. EPPO Aromia bungii found for the first time in Lombardia region, Italy. EPPO Reporting Service, 2013/187 (2013).
11. EPPO New additions to the EPPO A1 and A2 Lists. EPPO Reporting Service, 2014/158 (2014).
12. Regulation (EU) 1702 Supplementing regulation 2016/2031 of the European Parliament and of the Council by establishing the list of priority pests. Official Journal of the European Union. I. 260/8-10 (2019).
13. Carella, D. Regional Decree, DRD N°134 del 18/11/2019. Piano d'azione regionale per la lotta al cerambice cinese Aromia bungii. 7° aggiornamento (2019).
14. EPPO Update on the situation of Aromia bungii in Italy. EPPO Reporting Service no. 09 - 2017 (2017).
15. Azzoni, A. Definizione dell'area delimitata per la presenza di Aromia bungii (Faldermann) in Lombardia e applicazione delle misure fitosanitarie di contenimento; D.d.u.o. 21 may 2019 - n. 7119 (2019).
16. EPPO Aromia bungii found again in Bayern, Germany. EPPO Reporting Service no. 03 - 2017 (2017).
17. Tischner, H. Allgemeinverfügung der Bayerischen Landesanstalt für Landwirtschaft (LfL) über Maßnahmen zur Bekämpfung des Asiatischen Moschusbockkäfers (Aromia bungii Faldermann); vom 15.04.2019, Az. IPS 4c-7322.462 (2019).
18. EPPO First report of Aromia bungii in Japan. EPPO Reporting Service, 2013/188 (2013).
19. Kiriyama, S., Iwata, R. & Kagaya, E. Newly discovered populations of Aromia bungii (Faldermann), an invasive cerambycid infesting cherry and Japanese apricot trees in Tatebayashi, Gunma Pref. and Fussa, Tokyo Pref. *Plant. Prot.* **69**, 807–809 (2015).
20. Sugimoto, S. Collection report of Aromia bungii at Sayama Osaka Pref. *Gekkan-Mushi* **535**, 50–5 (2015).
21. EPPO Aromia bungii. EPPO Bull **45**, 4–8, https://doi.org/10.1111/epp.12173 (2015).
22. Haack, R. A., Hérard, F., Sun, J. & Turgeon, J. J. Managing Invasive Populations of Asian Longhorned Beetle and Citrus Longhorned Beetle: A Worldwide Perspective. *Annu. Rev. Entomol.* **55**(1), 521–546, https://doi.org/10.1146/annurev-ento-112408-085427 (2010).
23. Faccoli, M., Favaro, R., Smith, M. T. & Wu, J. Life history of the Asian longhorn beetle Anoplophora glabripennis (Coleoptera Cerambycidae) in southern Europe. *Agr. Forest. Entomol.* **17**, 188–196, https://doi.org/10.1111/afe.12096 (2015).
24. Duffy, E.A.J. A monograph of the immature stages of Oriental timber beetles (Cerambycidae). London: British Museum (Natural History), 434 pp (1968).

25. Zhang, X., Zeng, C. & Zhang, J. Bionomics and control techniques of Aromia bungii. *For. Pest. Dis.* **19**, 9–11 (2000).
26. Hu, C. X., Ding, Y. H. & Sun, K. Research advances of Aromia bungii in China. *Agric. Tech.* **27**, 63–67 (2007).
27. Garonna, A. P., Nugnes, F., Espinosa, B., Griffo, R. & Benchi, D. Aromia bungii, nuovo tarlo asiatico ritrovato in Campania. *Inf. Agrar.* **69**, 60–62 (2013).
28. Ma, W. H., Sun, L. Y., Yu, L. G., Wang, J. T. & Chen, C. Y. (2007) Study on the occurrence and life history in Aromia bungii (Faldermann). *Acta Agric. Boreali Sinica* **22**, 247–249 (2007).
29. Di Palma, A., Pistillo, M., Griffo, R., Garonna, A. P. & Germinara, G. S. Scanning electron microscopy of the antennal sensilla and their secretion analysis in adults of Aromia bungii (Faldermann, 1835) (Coleoptera, Cerambycidae). *Insects* **10**, 88, https://doi.org/10.3390/insects10040088 (2019).
30. Germinara, G. S., Pistillo, M., Griffo, R., Garonna, A. P. & Di Palma, A. Electroantennographic responses of Aromia bungii (Faldermann, 1835) (Coleoptera, Cerambycidae) to a range of volatile compounds. *Insects* **10**, 274, https://doi.org/10.3390/insects10090274 (2019).
31. Fukaya, M., Kiriyama, S. & Yasui, H. Mate location flight of the rednecked longicorn beetle, Aromia bungii (Coleoptera: Cerambycidae): an invasive pest lethal to Rosaceae trees. *Sci. Rep.* **7**, 7330, https://doi.org/10.1038/s41598-017-07520-1 (2017).
32. Men, J. *et al*. Behavioral responses of adults of Dastarcus helophoroides (Coleoptera: Bothrideridae) populations originated from different hosts to larval frass of Aromia bungii (Coleoptera: Cerambycidae) and their control effect on A. bungii population. *Acta Entomol. Sin.* **60**(2), 229–236 (2017).
33. Xu, T. *et al*. Identification of a male-produced sex-aggregation pheromone for a highly invasive cerambycid beetle, Aromia bungii. *Sci. Rep.* **7**, 7330, https://doi.org/10.1038/s41598-017-07520-1 (2017).
34. Zou, Y. *et al*. Optimizing pheromone-based lures for the invasive red-necked longhorn beetle, Aromia bungii. *J. Pest. Sci.* **92**, 1217, https://doi.org/10.1007/s10340-019-01108-6 (2019).
35. Wang, J. T., Sun, L., Liu, T. & Zhang, L. Research on the occurrence character and control measure of Aromia bungii. *J. Hebei. Agr. Sci.* **11**(2), 41–43 (2007).
36. Dlugosch, K. M. & Parker, M. Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Mol. Ecol.* **17**, 431–449, https://doi.org/10.1111/j.1365-294X.2007.03538.x (2008).
37. Valade, R. *et al*. Mitochondrial and microsatellite DNA markers reveal a Balkan origin for the highly invasive horse-chestnut leaf miner Cameraria ohridella (Lepidoptera, Gracillariidae). *Mol. Ecol.* **18**(16), 3458–3470, https://doi.org/10.1111/j.1365-294X.2009.04290.x (2009).
38. Nugnes, F. *et al*. Aleurocanthus spiniferus (Hemiptera: Aleyrodidae) in Europe: diffusion, hosts, molecular characterization, and its natural enemies. *Insects* **11**, 42, 0.3390/insects11010042 (2020).
39. Gillespie, R. G. & Roderick, G. K. Evolution: geology and climate drive diversification. *Nature* **509**, 297–298 (2014).
40. Bernardo, U. *et al*. Characterization, distribution, biology and impact on Italian walnut orchards of the invasive North-American leafminer Coptodisca lucifluella (Lepidoptera: Heliozelidae). *B. Entomol. Res.* **105**, 210–224, https://doi.org/10.1017/S0007485314000947 (2015).
41. Nugnes, F. *et al*. Genetic diversity of the invasive gall wasp Leptocybe invasa (Hymenoptera: Eulophidae) and of its Rickettsia endosymbiont, and associated sex-ratio differences. *PLOS ONE*, https://doi.org/10.1371/journal.pone.0124660 (2015).
42. Myers, J. H., Simberloff, D., Kuris, A. & Carey, J. R. Eradication revisited: dealing with exotic species. *Trends Ecol. Evol.* **15**, 316–320 (2000).
43. Huang, P., Yu, D., Yao, J., Wang, J. & Fang, D. Identification and damages of three kinds of Longicorn as well as their synthetical prevention on plum trees. *Biol. Disaster Sci.* **35**(1), 97–101 (2012).
44. Slipinski, S. A. & Escalona, H. E. Australian Longhorn Beetles Coleoptera: Cerambycidae Introduction and Subfamily Lamiinae. Csiro publishing. 2013; (i-xviii), pp. 484 (2013).
45. Monné, M. L., Monné, M. A. & Wang, Q. General morphology, classification and biology of Cerambycidae. In: Wang, Q (Eds.), Cerambycidae of the World: Biology and Pest Management. CRC Press, Boca Raton, FL, pp. 40–46 (2017).
46. Demelt, K. B, oder Cerambycidae. II. Part I. 52. Teil Tierwelt Deutschlands, Fischer Verlag, Jena, 115 pp (1966).
47. Hanks, L. M. Influence of the larval host plant on reproductive strategies of cerambycid beetles. *Annu. Rev. Entomol.* **44**, 483–505 (1999).
48. Duffy, E. A. J. A contribution towards the biology of Aromia moschata L., the musk beetle. *Proc. Trans. Br. Ent. Nat. Hist. Soc.* **1947–1948**, 82–110 (1949).
49. Mazaheri, A., Hatami, B., Khajehali, J. & Sadeghi, S. E. Reproductive parameters of Aeolesthes sarta Solsky (Col., Cerambycidae) on Ulmus carpinifolia Borkh. under laboratory conditions. *JAST* **11**, 333–343 (2007).
50. Sharifi, S., Javadi, J. & Chemsak, J. A. Biology of the Rosaceae Branch Borer, Osphranteria coerulescens (Coleoptera: Cerambycidae). *Ann. Entomol. Soc. Am.* **63**(6), 1515–1520 (1970).
51. Torres-Vila, L. M., Mendiola-Diaz, F. J., Conejo-Rodríguez, Y. & Sánchez-González, Á. Reproductive traits and number of matings in males and females of Cerambyx welensii (Coleoptera: Cerambycidae) an emergent pest of oaks. *Bull. Entomol. Res.* **106**, 292–303 (2016).
52. Lu, W. *et al*. Reproductive Traits of Glenea cantor (Coleoptera: Cerambycidae: Lamiinae). *J. Econ. Entomol.* **106**(1), 215–220, https://doi.org/10.1603/EC12251 (2013).
53. Togashi, K. & Yamashita, H. Effects of female body size on lifetime fecundity of Monochamus urussovii (Coleoptera: Cerambycidae). *Appl. Entomol. Zool.* **52**, 79–87 (2016).
54. Torres-Vila, L. M. Reproductive biology of the great capricorn beetle, Cerambyx cerdo (Coleoptera: Cerambycidae): a protected but occasionally harmful species. *Bull. Entomol. Res.* **107**(6), 799–811, https://doi.org/10.1017/S0007485317000323 (2017).
55. Togashi, K. Lifetime fecundity and female body size in Paraglenea fortunei (Coleoptera: Cerambycidae). *Appl. Entomol. Zool.* **42**, 549–556 (2007).
56. Keena, M. A. Anoplophora glabripennis (Coleoptera: Cerambycidae) fecundity and longevity under laboratory conditions: comparison of populations from New York and Illinois on Acer saccharum. *Environ. Entomol.* **31**, 490–498, https://doi.org/10.1603/0046-225X-31.3.490 (2002).
57. Togashi, K., Appleby, J. E., Oloumi-Sadeghi, H. & Malek, R. B. Age-specific survival rate and fecundity of adult Monochamus carolinensis (Coleoptera: Cerambycidae) under field conditions. *Appl. Entomol. Zool.* **4**, 249–256 (2009).
58. Leather, S.R. Factors affecting fecundity, fertility, oviposition, and larviposition in insects. Insect reproduction. Edited by: Leather SR, Hardie J., Boca Raton, CRC Press, 143–174 (1995).
59. Marco, V., Taberner, A. & Castañera, P. Development and survival of immature Aubeonymus mariaefranciscae (Coleoptera: Curculionidae) at constant temperatures. *Ann. Entomol. Soc. Am.* **90**, 169–176 (1997).
60. Zilahi-Balogh, G. M., Salom, S. M. & Kok, L. T. Temperature-dependent development of the specialist predator Laricobius nigrinus Fender (Coleoptera: Derodontidae). *Environ. Entomol.* **32**, 1322–1328 (2003).
61. Keena, M. A. Effects of temperature on Anoplophora glabripennis (Coleoptera: Cerambycidae) adult survival, reproduction and egg hatch. *Environ. Entomol.* **35**, 912–921 (2006).
62. García-Ruiz, E., Marco, V. & Pérez-Moreno, I. Effects of variable and constant temperatures on the embryonic development and survival of a new grape pest, Xylotrechus arvicola (Coleoptera: Cerambycidae). *Environ. Entomol.* **40**, 939–947 (2011).
63. Wiklund, C. & Persson, A. Fecundity, and the relation of egg weight variation to offspring fitness in the speckled wood butterfly Pararge aegeria, or why don't female butterflies lay more eggs? *Oikos* **40**(1), 53–63 (1983).

64. Rosenheim, J. A., Heimpel, G. E. & Mangel, M. Egg maturation, egg resorption and the costliness of transient egg limitation in insects. *P. Roy. Soc. B-Biol. Sci.* **267**, 1565–1573 (2000).
65. Haack, R. A., Keena, M. A. & Eyre, D. Life history and population dynamics of cerambycids. In: Wang Q (Eds) Cerambycidae of the World: Biology and Pest Management. CRC Press, Boca Raton, pp 71-103, 10.1201/b21851 (2017).
66. Bybee, L. F., Millar, J. G., Paine, T. D., Campbell, K. & Hanlon C. C. Effects of temperature on fecundity and longevity of Phoracantha recurve and P. semipunctata (Coleoptera: Cerambycidae). *Environ. Entomol.* 33, 138–46, https://doi.org/10.1603/0046-225X-33.2.138 (2004).
67. Chapman, T., Miyatake, T., Smith, H. K. & Partridge, L. Interactions of mating, egg production and death rates in females of the Mediterranean fruit fly, Ceratitis capitata. *Proc. Biol. Sci.* **265**, 1879–1894, https://doi.org/10.1098/rspb.1998.0516 (1998).
68. Kotiaho, J. S. & Simmons, L. W. Longevity cost of reproduction for males but no longevity cost of mating or courtship for females in the male dimorphic dung beetle Onthophagus binodis. *J. Insect. Physiol.* **49**, 817–822 (2003).
69. Wilson, K. & Hardy, I. C.W. Statistical analysis of sex ratios: an introduction in Hardy ICW, editor. In: Sex Ratios Concepts and Research Methods. [Cambridge]: Cambridge University Press. Pp. 404. (2002).
70. Matsushita, M. B. zur Kenntnis der Cerambyciden des japanischen Reichs. *J. Fac. Agric. Hokkaido Imp. Univ.* **34**(2), 157–445 (1933).
71. Gebiola, M., Bernardo, U., Monti, M. M., Navone, P. & Viggiani, G. Pnigalio agraules (Walker) and Pnigalio mediterraneus Ferrière and Delucchi (Hymenoptera: Eulophidae): two closely related valid species. *J. Nat. Hist.* **3**, 2465–2480, https://doi.org/10.1080/00222930903105088 (2009).
72. Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. DNA primers for amplification of mitochondrial cyto chrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotech.* **3**, 294–299 (1994).
73. Campbell, B. C., Steffen-Campbell, J. D. & Werren, J. H. Phylogeny of the Nasonia species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. *Insect. Mol. Biol.* **2**, 225–237, https://doi.org/10.1111/j.1365-2583.1994.tb00142.x (1993).
74. Hall, T. A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acid S.* **41**, 95–98 (1999).
75. Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* **30**, 2725–2729, https://doi.org/10.1093/molbev/mst197 (2013).
76. Jervis, M. A., Heimpel, G. E., Ferns, P. N., Harvey, J. A. & Kidd, N. C. Life-history strategies in parasitoid wasps: a comparative analysis of "ovigeny". *J. Anim. Ecol.* **70**, 442–458, https://doi.org/10.1046/j.1365-2656.2001.00507.x. (2001).
77. Statgraphics plus Version 3.0. Manugistics. Maryland, USA (1997).

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Author contributions

E.R., F.N. and U.B. conceived and designed research. E.R., F.N., U.B. and A.P.G. conceptualized the methodology. U.B., F.N. and E.R. performed statistical analyses. E.R., F.N. and F.V. carried out field investigations. F.N., E.R. and F.V. conducted laboratory tests. E.R., F.N. and U.B. wrote the original draft. E.R., F.N., U.B. and A.P.G. wrote and edited the manuscript. U.B. and A.P.G. coordinated the project. U.B. provide the funds. All authors read and approved the manuscript.

Competing interests

The authors declare no competing interests.

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The establishment of a rearing technique for the fruit fly parasitoid *Baryscapus silvestrii* increases knowledge of biological, ecological and behavioural traits

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Abstract We have evaluated different rearing strategies of *Baryscapus silvestrii* (Hymenoptera: Eulophidae), a parasitoid of *Bactrocera oleae* (Diptera: Tephritidae), including the use of a factitious host, *Ceratitis capitata* (Diptera: Tephritidae), and in the process acquired new knowledge of the parasitoid's biology. We found that *B. silvestrii*: (1) parasitizes only puparia and exclusively if they are concealed, (2) is able to parasitize and complete its development on puparia of all ages, (3) prefers to oviposit on the medfly in choice tests, (4) completes development faster if reared on 2–3 day-old puparia, (5) exhibits sex allocation related to host puparium age. This study provides critical information on several biological traits of *B. silvestrii*, and the new rearing method can be used to establish a parasitoid rearing for augmentative releases.

Keywords Biological control · *Bactrocera oleae* · *Ceratitis capitata* · Eulophidae · Sex allocation

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Introduction

Fruit flies represent one of the biggest threats to agriculture worldwide due to damages caused by both adults and larvae. They are considered so dangerous that the mere presence in some areas prevents free trade between countries and triggers costly quarantine procedures (Shelly et al. 2014), as it is currently happening in Italy after the finding of just seven specimens of *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) in field traps (Nugnes et al. 2018). The Mediterranean fruit fly or medfly, *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) and the olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), are among the most important and studied species (Rice et al. 2003; Daane and Johnson 2010; Ekesi et al. 2016). Due to its high adaptability and invasive

capacity, *C. capitata* is one of the main global pests of fruit trees, in particular *Citrus* spp. (Zucchi 2001). The medfly is multivoltine, highly polyphagous, as the host range comprises more than 260 species (Liquido et al. 1991), and displays different host preferences in different regions of the world. The medfly is native to sub-Saharan Africa but it is currently established throughout Africa, European countries adjacent and proximal to the Mediterranean Sea, the Hawaiian Islands, the Middle East, and Central and South America. Its worldwide economic costs can amount to many US$ billions each year due to direct and indirect damages (Szyniszewska and Tatem 2014).

The olive fruit fly, *B. oleae*, is a major pest to olive throughout the Mediterranean basin, South and Central Africa, Canary Islands, the Near and Middle East, USA, Central and South America (Daane and Johnson 2010; Malheiro et al. 2015). Larvae are monophagous on the fruit of several species in the genus *Olea*. The magnitude of damages caused by *B. oleae* is considerable, often leading to a reduction of at least 15% of the global olive production (Malheiro et al. 2015), although a correct timing of harvest and a quick extraction from olives bearing up to 40% or 45% of infestation can still produce high quality extra virgin olive oil (Gucci et al. 2012, Caleca et al. 2017).

Both fruit flies need the application of control measures, and given the European policy of banning harmful pesticides (Storck et al. 2017), the use of natural enemies in IPM strategies is becoming essential. Biological control of fruit flies has a very long history that started with the founder of the entomological school in Portici, Filippo Silvestri, who released many African parasitoids in Italy (Silvestri 1913, 1915). Although populations of several fruit flies have been reduced by the introduction of parasitoids (Harris et al. 2010; Ekesi et al. 2016), biological control of *C. capitata* and *B. oleae* in the Mediterranean basin, and *B. oleae* in USA has not reached a satisfactory level (Headrick and Goeden 1996; Daane et al. 2015). Until 2007, only five parasitoids were commonly reared from *B. oleae* in Europe: the chalcid wasps *Cyrtoptyx latipes* (Rondani) (Hym.: Pteromalidae), *Eupelmus urozonus* Dalman (Hym.: Eupelmidae), *Eurytoma martellii* Domenichini (Hym.: Eurytomidae), *Pnigalio mediterraneus* Ferrierre & Delucchi (Hym.: Eulophidae), and the Braconid *Psyttalia concolor* (Szépligeti) (Bernardo and Guerrieri 2011; Hoelmer et al. 2011). After the establishment of *B. oleae* in California, USA (Rice et al. 2003), there was a renewed interest in classical biological control of this pest and much attention was focused on the *P. concolor* species complex from sub-Saharan Africa. However, this parasitoid complex does not provide effective biological control (Bigler et al. 1986; Johnson et al. 2012), therefore the prospect of having another parasitoid is appealing.

In 2007, a gregarious pupal parasitoid of *B. oleae*, the eulophid *Baryscapus silvestrii* Viggiani and Bernardo (Hymenoptera: Eulophidae) was described (Viggiani et al. 2007). Since then, this species has expanded its distribution range in Italy in Campania and Lazio regions, becoming predominant and has been also found in Sicily (Bernardo, personal observation). However, its biology remains largely unknown, due to several failures at establishing a laboratory rearing on the olive fruit fly (Viggiani et al. 2007). Nevertheless, it is clear that *B. silvestrii* adults emerge from host pupae, and usually pupal parasitoids are idiobionts that kill quickly by venom or by host feeding their hosts. Successively, the immature parasitoids feed and develop on the host carcass. The nutritional resources are, therefore, determined solely by the size of the parasitized host stage.

The main aim of this research was to develop a methodology to establish a permanent rearing of *B. silvestrii* using *C. capitata* as host. The whole process could provide new knowledge on biological parameters and on the potential of this parasitoid as a biocontrol agent of fruit fly pests.

Materials and methods

Rearing of *C. capitata*

Adult flies were maintained in a Bugdorm-1 cage (30 × 30 × 30 cm "BioQuip products") at a density of about 300 adult medflies per cage. Adult flies had ad libitum access to water in a glass vial covered with a cotton plug and 3:1 dry mixture sucrose and yeast extract placed in a flat container. Females laid eggs through the net walls of the cages that were placed on four supports (8 × 6 × 6 cm) in a rectangular water-filled vessel (60 × 70 cm). Daily, eggs not fallen in the vessel were detached from the net with the help of a brush and collected (Pašková 2007). Adult laboratory colonies were maintained in a temperature-

controlled climatic chamber (25 ± 1 °C, L:D 16:8 photoperiod, 60 ± 10% RH) in two laboratories. Collected eggs were placed on 50 ml of a solidified artificial diet modified from that of Pašková (2007), consisting of 2 g nipagin, 4 g agar, 100 g of yeast, 50 g of sucrose, and 600 ml of water in Petri dishes (ø 9 cm). Petri dishes were placed in a climatic chamber at 25 ± 1 °C, L:D 16:8 photoperiod, 60 ± 10% RH. After about nine days, the Petri dishes were placed in plastic boxes, with sterile river sand on the bottom, shortly before pupation. Mature larvae jumped out of the diet and pupated on the substrate, from which puparia were daily collected.

Suitability of different developmental stages of *C. capitata* as hosts

A laboratory colony of *B. silvestrii* was set up with adults emerged from puparia of *B. oleae* collected in October 2014 from infested olive fruits in Portici (40° 48′ 54.16″ N and 14° 20′ 51.92″ E, 77 m a.s.l.), Italy.

Bioassay A1: suitability of host larval stages

No-choice tests were carried out to assess the parasitism preference of *B. silvestrii* for *C. capitata* larvae. Ten replicates were carried out. Five *C. capitata* larvae of each age cohort (i.e., 2nd and 3rd instar) were exposed to a single couple (one male and one female) of three days old parasitoids in the oviposition chamber, consisting of a 200 ml plastic cup with a fine mesh top for ventilation. Larvae were placed on the bottom of each unit that contained ad libitum honey as a nutritional source for the parasitoids. The parasitism units, in which larvae of *C. capitata* were offered to parasitoids, were provided with side muslin lids through which larvae of *C. capitata* were offered to parasitoids. After 24 h, larvae were collected and placed inside ventilated Petri dishes that were kept in the climatic chamber to allow completion of development of both medflies and parasitoids. Larvae of the second instar developed in puparia and then into smaller flies also in the absence of food. After the flies' emergence, the remaining puparia were kept in glass vials (10 cm long, 1 cm ø, with a cotton cover) and checked daily to record the emergence time (i.e., days from egg to adult emergence from the host puparium), the number and sex of emerging wasps. Due to the peculiar behaviour of males before emergence (males wait for the emergence of females inside the puparia to mate with them), the emergence time of males and females were analysed separately.

Bioassay A2: suitability of host pupal stages

No-choice tests were carried out to assess the parasitism preference of *B. silvestrii* for *C. capitata* puparia. To examine the preference of female wasps for host puparia of different ages, ten *C. capitata* puparia of each different age cohorts (0–7 day-old) were exposed to parasitoids as reported for Bioassay A1. The emergence time, number and sex of parasitoids were recorded. A total of ten replicates were carried out.

Bioassay A3: suitability of host larvae and pupae inside fruits

To reproduce the natural microhabitats, and due to the failure of the previous bioassays (see Results), larvae and puparia inserted inside fruits were offered to parasitoids in the same conditions of the no-choice tests reported in Bioassays A1 and A2. One slice of apple (var. *Golden delicious*) was artificially infested. An incision similar to a deposition puncture of fly, with the typical deposition chamber, was made with a scalpel blade (6 × 5 mm). The apple epidermis was raised to insert larvae or puparia and then rearranged (ten holes per slice, one for each larva or puparium). Ten 2nd or 3rd instar larvae or ten puparia (0–7 day-old) of *C. capitata* were used in each test. Artificially infested apple slices were placed in the parasitism units (25 ± 1 °C, L:D 16:8 photoperiod, 60 ± 10% RH). Three 3–5 day-old *B. silvestrii* couples were placed for 24 h inside parasitism unit each containing honey as nutritional sources. Then, medfly larvae and puparia were recovered and put in ventilated Petri dishes, kept in the climatic chamber, until the emergence of medfly adults. After the flies' emergence, the remaining puparia were kept in glass vials (10 cm long, 1 cm ø, with a cotton cover) and checked daily to record the emerging parasitoid wasps. The presence of parasitoid offspring was recorded.

Host preference tests

Based on Bioassay A3 (see Results), the pieces of apple were replaced with pleated paper cardboards in order to simplify the rearing and to recreate a narrow, tight and concealed environment, as preferred by *B. silvestrii* adults. Choice and no-choice tests were carried out to assess the parasitism preference of *B. silvestrii*.

Bioassay B1: preference for puparia of C. capitata or B. oleae

A couple of *B. silvestrii* reared on *C. capitata* was exposed simultaneously to five three days old *C. capitata* and five three days old *B. oleae* puparia at the same conditions reported above (n = 21). Medfly puparia were taken from the established permanent rearing, while *B. oleae* puparia were reared daily from olive fruits collected in an organic orchard. After 24 h, exposed puparia were placed singly in glass vials (10 cm long, 1 cm ø, with a cotton cover) and checked daily to record the number of parasitized puparia and offspring sex.

Bioassay B2: preference for pupal ages of C. capitata

Forty *C. capitata* puparia of eight different age cohorts (0–7 day-old) (five puparia per age) placed in pleated paper cardboards (a line per age) were offered to parasitoids for 24 h to identify the puparium age preferred by the wasps. In each replicate (n = 32) the order of different puparia age shifted over a line on the pleated paper cardboards, hence each puparium age occupied every position on the paper cardboard. Replicates were discarded if no parasitization occurred. Each parasitoid was permitted to parasitize a single time, and discarded after the first choice.

Bioassay C: host pupal suitability for parasitoid development and developmental parameters

To determine the most suitable pupal host age for parasitoid development, a parasitoid couple of the same age (one day old) was held for 24 h in an oviposition chamber with ten puparia of *C. capitata* of the same age on a pleated paper. All different eight age puparia (0–7 days old) were tested on a pleated paper. Puparia were replaced daily. Adult parasitoids were fed with honey. Any male parasitoid that died before the end of the test was replaced with newly emerged males. Ten replicates were performed. Synchronized cohorts of different stages of host puparia were obtained by collecting daily newly pupated *C. capitata* from laboratory colonies and keeping them separate in an oviposition chamber dedicated to each age class (0–7 days old) until use. Each test was stopped when 25 males and 25 females parasitoid offspring emerged from the first parasitized puparia. After exposure, puparia were transferred to incubation units, 100 ml plastic cups (4.5 cm diameter, 5.5 cm depth) with perforated cap, until emergence of adult flies. Then the remaining puparia were kept individually in glass vials and checked daily to record the number and sex of emerging parasitoids. The emergence time, number and sex of emerged parasitoids and the number of exit holes on puparia were recorded.

Statistical analysis

Data satisfying conditions of normality and homoscedasticity, both untransformed or after appropriate transformation, were analysed by ANOVA, if not differently reported, and the means were separated at the 0.05 level of significance by a multiple range test (Tukey, or in case of unequal samples, Bonferroni). In all other cases, a non-parametric test (Kruskal–Wallis) was used, and medians were separated graphically by a Box and Whisker plot analysis (Statgraphics plus 1997).

Preference data were analysed by a χ^2 test at the 0.05 level of significance to evaluate data independence assuming that the choice of different pupal stages follows a Poisson distribution with a mean of 4.0 in Bioassay B1. Replicates with female parasitoids that survived the 24 h oviposition period of the choice tests, but did not parasitize any puparium, were excluded from further analysis. The statistical comparison of the double parasitized hosts (two host puparia) was made using the results of the bioassay C (see below) as control.

A percent males was computed and analysed by a logistic regression with binomial error distribution and a logit link function, followed, if necessary, by Tukey contrasts for multiple comparisons of means as implemented by the R package 'multcomp' (Kenneth and Hardy 2002). These statistical analyses were performed using R (R Core Team 2018).

The relationship between progeny and the number of holes in puparia hosts (bioassay C, no-choice tests) was assessed by regression analysis. All data are presented non-transformed with SE.

Results

Bioassay A1: suitability of larval stages of C. capitata

No progeny of *B. silvestrii* was obtained from this bioassay. Observations showed that females exhibited no interest in the larvae.

Bioassay A2: suitability of pupal stages of C. capitata

No progeny of *B. silvestrii* was obtained from this bioassay. Females were observed touching the host with the antennas and walking on the pupae but no attempt of oviposition was observed.

Bioassay A3: suitability of larvae and pupae inside fruits

No progeny of *B. silvestrii* was obtained from fruits infested with larvae. Progeny was recorded from fruits infested with puparia and parasitoid females were observed entering the holes and searching for the puparia under the fruit epidermis.

Choice tests

Bioassay B1: host species preference

Baryscapus silvestrii females parasitized both *C. capitata* and *B. oleae* and the mean progeny emerging from the puparia of both species was very similar (15.8 ± 2.22 and 11.9 ± 2.49 adults, respectively, ANOVA, $F_{1, 27} = 1.19$, p = 0.28). Parasitoid females chose to parasitize *C. capitata* puparia in 52.4% of instances, and *B. oleae* puparia in 9.5% of cases, and in 38.1% of cases parasitized one puparium per species. Females simultaneously exposed to puparia of both species parasitized a significantly higher number of puparia (1.55 ± 0.09, n = 29), than females exposed to a single species (1.06 ± 0.03 host puparia, n = 80) (6.67%) (Kruskal–Wallis, $\chi^2 = 32.45$, df = 1, $P < 0.001$).

Mean percentage of *B. silvestrii* adults emerged from *C. capitata* puparia resulted higher than those emerged from *B. oleae* puparia (74.8 ± 7.26 adults respect to 25.2 ± 7.26) (p = 0.003). Also, sex ratio of emerged adults resulted affected by host puparia because a higher number of males emerged by *C. capitata* puparia 0.40 ± 0.09 compared to *B. oleae* puparia 0.13 ± 0.03 (Wald test, $\chi^2 = 50.82$, df = 1, $P < 0.001$).

Bioassay B2: host pupal age preference

Females did not parasitize more than a single puparium per day. Thirty-two puparia were parasitized and parasitoid females stung puparia of any age with the exception of six days old puparia. *B. silvestrii* adults emerged from all parasitized puparia. Both males and females emerged from all the puparia, with the sole exception of the newly formed puparia from which only females emerged. *Baryscapus silvestrii* showed significant variation in its innate preference for the eight host puparia ages ($\chi^2 = 8.93$, df = 1, $P < 0.05$). Differences in parasitoid choice resulted significant in three cases (Table 1).

No-choice tests

Bioassay C: host pupal suitability and developmental parameters

Parasitoid females parasitized host puparia of any tested age. Progeny emerged from each puparium ranged from one to 45. However, some differences were observed, as is summarized in Fig. 1 (ANOVA, $F_{7, 72} = 2.40$, $P < 0.05$). Statistical differences are due to female offspring (ANOVA, $F_{7, 72} = 2.42$, $P < 0.05$), because male offspring did not vary across puparia age (Kruskal–Wallis, $\chi^2 = 11.17$, df = 7, p = 0.131). Females developing on two or three days old puparia completed their development earlier than females developing on puparia of other ages, and females emerged from five days old puparia showed the longest emergence time (Kruskal–Wallis, $\chi^2 = 119.56$, df = 7, $P < 0.05$) (Fig. 2). Emergence time of males reared on puparia of different ages resulted statistically different (Kruskal–Wallis, $\chi^2 = 41.28$, df = 7, $P < 0.05$) (Fig. 2), although with a lower variability than females, and due to sib-mating, male emergences was surely affected by their

Table 1 Innate host preference of *B. silvestrii* determined from host acceptance when all hosts were exposed simultaneously (five puparia per each puparia stage 0–7 days old) (n = 32)

Pupal age (days)	Observed choices	Expected choices	P value	Observed preference
0	2	4	0.1465	0
1	2	4	0.1465	0
2	2	4	0.1465	0
3	12	4	0.0006	+
4	2	4	0.1465	0
5	4	4	0.1954	0
6	0	4	0.0183	−
7	8	4	0.0298	+

The P-values indicate the significance level of the differences between the expected and the observed choices for each pupal age
Observed preference: +, more preferred; 0, neutral; −, less preferred

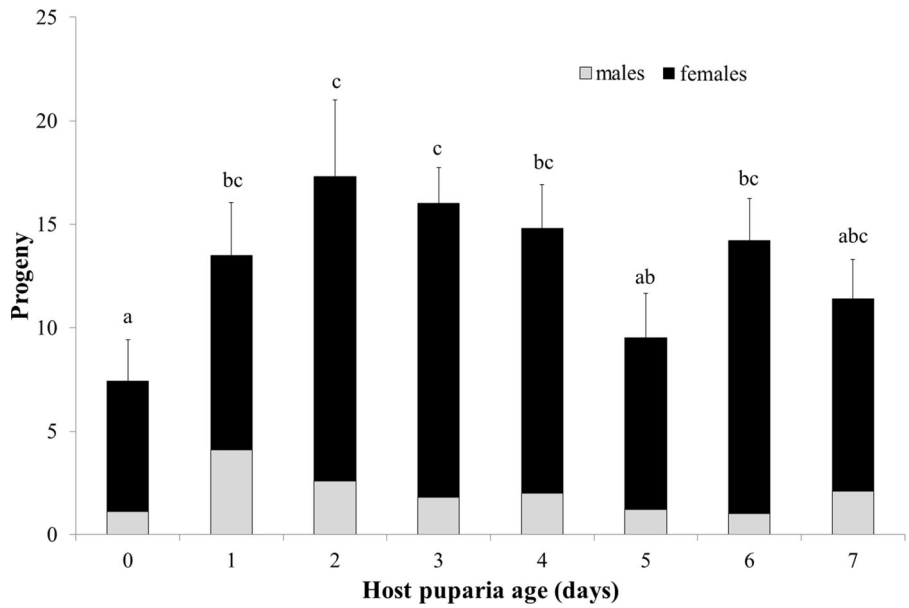

Fig. 1 Mean progeny (+ SE) of *B. silvestrii* reared on puparia of different age. Bars with the same letter are not significantly different at the 5% level (ANOVA). Data were analysed as total progeny (males + females)

waiting of female into puparia. Emergence holes per puparium ranged between one and four and a statistical difference was only recorded between 0, five days old puparia and two days old puparia (1.2 ± 0.20 and 1.4 ± 0.26 respect to 2.7 ± 0.30) (ANOVA, $F_{7,\ 51} = 3.79$, $P < 0.005$) (Fig. 3). The number of holes was weakly correlated to the progeny ($r = 0.206$, $F_{1,\ 57} = 16.08$; $P < 0.001$). Sex ratio ranged between 0.13 and 0.26 ($\chi^2 = 141.32$, df = 7, $P < 0.001$) (Fig. 4).

Discussion

The development of an easy and inexpensive parasitoid rearing method is a critical step in biocontrol programs. *B. silvestrii* has proved to be a species with peculiar behavioural and biological traits that have made the establishment of a mass rearing very difficult. Viggiani et al. (2007) stated that it would be impossible to rear this parasitoid. However, we have now managed to overcome most difficulties, and this study shows the great potential of puparia of the

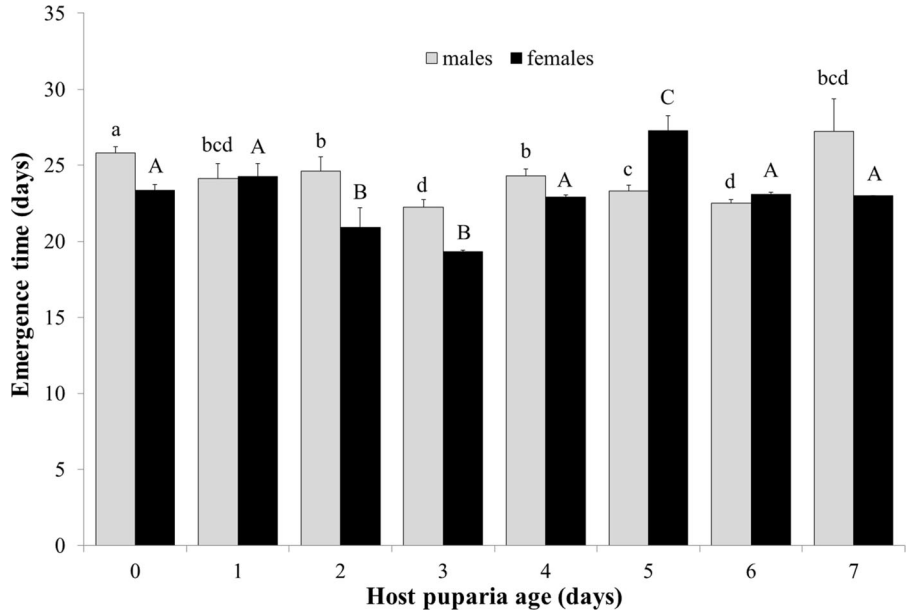

Fig. 2 Emergence time (mean + SE) of males and females of *B. silvestrii* reared on puparia of different age. Emergence time of males and females were analysed separately due to the peculiar behaviour of males. Bars with the same capital letter (females) or lower case (males) are not significantly different at the 5% level (Kruskal–Wallis)

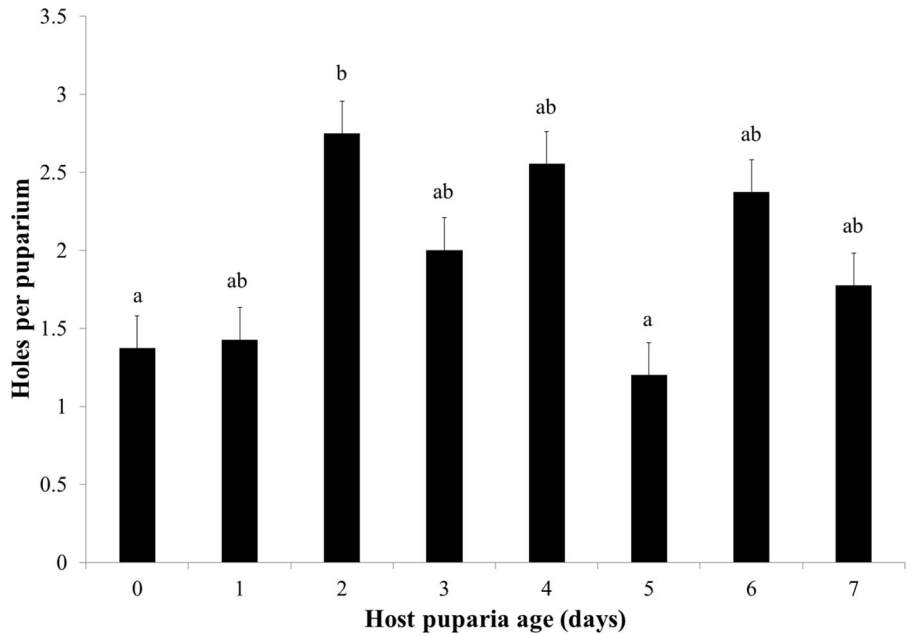

Fig. 3 Emergence holes (mean + SE) made by *B. silvestrii* adults per puparium reared on puparia of different age. Bars with the same letter are not significantly different at the 5% level (ANOVA)

medfly *C. capitata* as factitious host for the mass rearing of *B. silvestrii*. The tests here carried out allowed establishing that *B. silvestrii* develops as primary endoparasitoid and has an endophagous and gregarious behaviour parasitizing exclusively puparia. The oviposition behaviour of *B. silvestrii* is truly peculiar, as no *C. capitata* puparia are parasitized if they are not at least partly concealed, in fruits or in

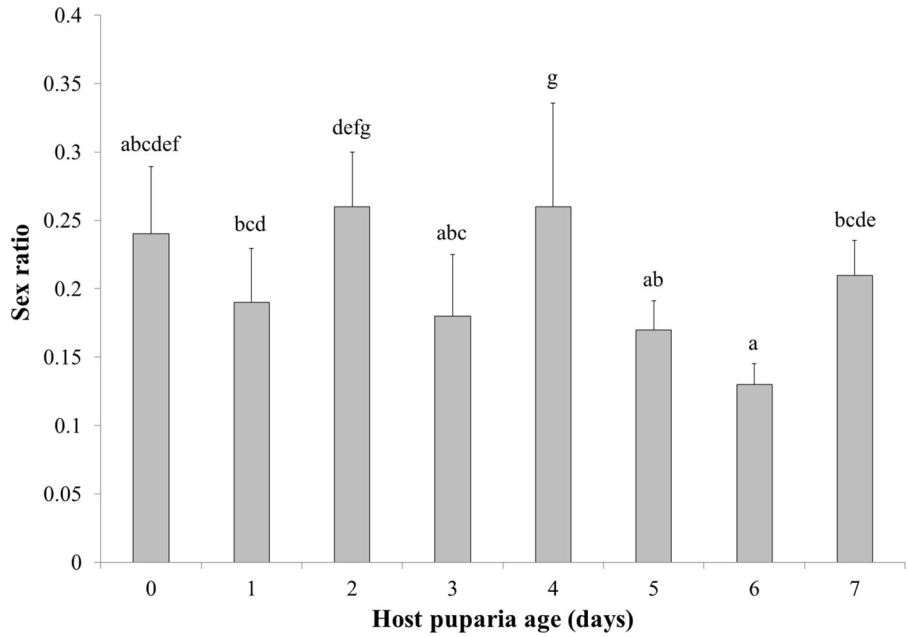

Fig. 4 Sex ratio (mean + SE) of progeny reared on puparia of different age. Bars with the same letter are not significantly different at the 5% level

pleated paper cardboards and although the last solution may seem a little laborious, pleated paper cardboards can be recycled many times, and eliminating the need for fruit supply allows an easier parasitoid rearing, since it avoids parasites (e.g., mites, flies) and rotting, and minimize costs.

Choice tests between the factitious host (*C. capitata*) and the primary host (*B. oleae*) showed a preference of the parasitoids for the puparia of *C. capitata*. However, this behaviour may have been strongly influenced by the natal rearing host, *C. capitata*, because usually female of parasitoids with a wide host range often prefer the host species on which they have been reared (Davis and Stamps 2004).

Although the medflies' puparia are larger than those of *B. oleae,* the mean progeny per female was unaffected by the host species, differently from what occurs in other parasitoids (Vinson 1976). However, the sex ratio of the progeny emerged from *C. capitata* was less female-biased than the ones from *B. oleae* (proportion of males = 0.40 vs. 0.13), ascribable to a possible sex allocation preference. Females of *B. silvestrii* do not seem to prefer laying male eggs in smaller hosts (*B. oleae*), in contrast to other parasitoid wasps (Jones 1982, King 1988; Napoleon and King 1999), but this behaviour could also depend on the lower suitability of the factitious host, *C. capitata*.

The peculiar mating behaviour of the parasitoid may have affected our results. *B. silvestrii* showed protandry, since males emerged before the females and waited for a female to mate inside the puparium, leading to within-host sib-mating. This type of mating system is frequent in gregarious and quasi-gregarious parasitoids (Heimpel and Lundgren 2000; Boulton et al. 2015) and may reduce the necessity of high depositions of male eggs in qualitatively inferior hosts, because fecundation is guaranteed by a smaller number of males. However, another possible explanation is that the mate competition among males waiting for females' emergence inside the puparium results in a female biased sex ratio, as it happens for example in fig wasps (Cook et al. 1997).

Sib-mating determines high consanguinity that does not seem to cause problems to *B. silvestrii* because even after over five years of laboratory rearing, no evident deterioration in fitness of this species has been recorded. This is another excellent feature of *B. silvestrii*, as it eliminates the need of periodically introducing new field-collected populations in the insectary, which might have unintended consequences, such as introducing misidentified species (see for example: de Almeida and Stouthamer 2003; Nugnes et al. 2017), if a thorough integrative

characterization has not been carried out and a rigid quality control protocol is not adopted.

Females of *B. silvestrii* usually show a low propensity in distributing the egg load over different puparia in a single day (6.67% of depositions in two host puparia), but when two puparia of different species are available, the frequency of double parasitization increases significantly, reaching 38.1%. To the best of our knowledge, this peculiar behavioural plasticity is reported here for the first time. Evidently, two hosts are better than one, and we suppose that females, having the availability of different hosts of comparable suitability, prefer to distribute eggs into two hosts to increase the odds of offspring survival.

Despite our results showed *C. capitata* is a suitable host, to date *B. silvestrii* have never been reared from its puparia collected in the field. A possible explanation could be that the larvae of *B. oleae* often remain in the drupes where they pupate, emerging directly as adults. Differently, mature larvae of *C. capitata* usually pupate in the ground, thus likely managing to escape parasitization by a selectivity of position (Holt and Lawton 1994). However it is noteworthy that, although the depth of pupation of many tephritids is related to several abiotic soil factors, usually they pupate very superficially in the ground (Jackson et al. 2008), remaining detectable and accessible to parasitoids. To give a definitive answer about the possibility that *B. silvestrii* could parasitize puparia in the ground, specific experiments are needed.

Choice tests with different pupal ages of *C. capitata* showed that *B. silvestrii* prefers three and seven days old puparia, although all pupal ages in the interval 0–7 days were parasitized, with the exclusion of six days old puparia, and progeny emerged from puparia of each age. The reason for this lack of preference to six days old puparia is still unclear, hence further studies are required to explain if factors like physiological status, volatile organic compounds, tissue development, or water content are involved in the host puparia attractiveness to *B. silvestrii* females. *B. silvestrii*, therefore, can parasitize both newly developed puparia and late puparia (with completely developed imagoes), which makes this parasitoid particularly interesting because the presence of such large window of host vulnerability is a desirable feature for biological control.

No-choice tests with different pupal ages showed that *B. silvestrii* can develop on all puparia ages. However, the age of puparia affected both the emergence of parasitoids and their progeny influencing also the sex ratio of the offspring, which, although strongly female-biased, varies between 0.16 (in six days old puparia) and 0.26 (in four and two days old puparia) (Fig. 4). These results confirm the great adaptability of *B. silvestrii*, but are not consistent with sex ratio theory, specifically the host size model (i.e. a sex ratio response to host resources as measured by external host dimensions) according to which the parasitoids tend to allocate male eggs in smaller hosts (Charnov et al. 1981) although this theory has been developed for solitary parasitoid wasps.

From young (< 24 h) and five days old puparia fewer progeny has emerged if compared to the other stages. Moreover on five days old puparia, parasitoids showed the longest emergence time, therefore these stages seem to be the least suitable host stage for the rearing. Differently, parasitoids reared on 2–3 day-old puparia showed the shorter emergence time and a more numerous progeny. This combination and the fact that the three days old puparia are also the most parasitized in the choice tests highlight that the use of three days old puparia should guarantee the best conditions for mass rearing.

No-choice tests showed that parasitoid females parasitized host puparia of any tested age and that the suitability of different host pupal ages is minimal for five days old puparia (for all biological examined traits), but then slightly improves again in seven days old puparia (in terms of sex ratio). The importance of the host developmental stage is here clear, as at seven days the medfly pupa is an almost complete imago and, in this case, parasitoid females lay their eggs within the host abdomen of pharate adult. It is likely that the water content of pupae, which decreases through development, has an important role in the suitability of the different pupal ages. Therefore, conceivably puparia of 5–6 day-old are at a less suitable stage for the parasitoid development, which in turn determines both an increase of the emergence time and a lack of depositions recorded in the choice test in puparia of six days old puparia. The number of emergence holes, which is slightly affected by the host pupal age (excluding zero and five days old puparia, all others ages did not result different), seems to be slightly affected also by the number of emerging adults. A possible explanation for this parasitoid behaviour could be that throughout the intra-puparial

host development, which, for cyclorrhaphous Diptera, can be divided into four stages (larval-pupal apolysis, cryptocephalic pupa, phanerocephalic pupa, and pharate adult, Martín-Vega et al. 2016), the space inside the puparia is compartmentalized in different ways, thus making further exit points necessary for the adults that fail to reach those made by their siblings.

In conclusion, our data indicate that *B. silvestrii* has the potential to be considered a suitable biological control agent of *B. oleae* and the ability to parasitize *C. capitata*, as it can be easily reared on a factitious host (the medfly) and reproduced in large quantities for augmentative releases. However, more studies are necessary to understand better the advantages and limitations of this species as a natural enemy of fruit fly pests under field conditions, and to know others biological parameters (in particular the optimal temperature range for development) that could allow a comparison of this species with other parasitoids that are commercially available against fruit flies. The availability of a new commercial parasitoid of *B. oleae* would be extremely important because the current parasitoid complex does not provide effective control. Moreover, as *B. silvestrii* occupies a different ecological niche (it is a pupal idiobiont while most of the common parasitoids of *B. oleae* are koinobiont larval-pupal endoparasitoid), the intraguild competition should not be very strong.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

Bernardo U, Guerrieri E (2011) Controllo eco-sostenibile della mosca dell'olivo: recenti acquisizioni. Acta Italus Hortus 1:351–354

Bigler F, Neuenschwander P, Delucchi V, Michelakis S (1986) Natural enemies of preimaginal stages of *Dacus oleae* Gmel. (Dipt., Tephritidae) in Western Crete II. Impact on olive fly populations. Boll Lab Entomol Agrar F Silvestri 43:79–96

Boulton RA, Collins LA, Shuker DM (2015) Beyond sex allocation: the role of mating systems in sexual selection in parasitoid wasps. Biol Rev Camb Philos Soc 90:599–627

Caleca V, Antista G, Campisi G, Caruso T, Lo Verde G, Maltese M, Rizzo R, Planeta D (2017) High quality extra virgin olive oil from olives attacked by the olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera Tephritidae): which is the tolerable limit? Data from experimental 'Nocellara del Belice' and 'Cerasuola' olive groves in Sicily. Chem Eng Trans 58:451–456

Charnov EL, Los-den Hartogh RL, Jones WT, van den Assem J (1981) Sex ratio evolution in a variable environment. Nature 289:27–33

Cook JM, Compton SG, Herre EA, West SA (1997) Alternative mating tactics and extreme male dimorphism in fig wasps. R Soc B 264:747–754

Daane K, Johnson M (2010) Olive fruit fly: managing an ancient pest in modern times. Annu Rev Entomol 55:155–169

Daane KM, Wang X, Nieto DJ, Pickett CH, Hoelmer KA, Blanchet A, Johnson MW (2015) Classic biological control of olive fruit fly in California, USA: release and recovery of introduced parasitoids. BioControl 60:317–330

Davis JM, Stamps JA (2004) The effect of natal experience on habitat preferences. Trends Ecol Evol 19:411–416

de Almeida RP, Stouthamer R (2003) Molecular identification of *Trichogramma cacoeciae* Marchal (Hymenoptera: Trichogrammatidae): a new record for Peru. Neotrop Entomol 32:269–272

Ekesi S, De Meyer M, Mohamed SA, Virgilio M, Borgemeister C (2016) Taxonomy, ecology, and management of native and exotic fruit fly species in Africa. Annu Rev Entomol 61:219–238

Gucci R, Caruso G, Canale A, Loni A, Raspi A, Urbani S, Taticchi A, Esposto S, Servili M (2012) Qualitative changes of olive oils obtained from fruits damaged by *Bactrocera oleae* (Rossi). HortScience 47:301–306

Harris EJ, Bautista RC, Vargas RI, Jang EB, Eitam A, Leblanc L (2010) Suppression of melon fly (Diptera: Tephritidae) populations with releases of *Fopius arisanus* and *Psyttalia fletcheri* (Hymenoptera: Braconidae) in North Shore Oahu, HI, USA. BioControl 55:593–599

Headrick DH, Goeden RD (1996) Issues concerning the eradication or establishment and biological control of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), in California. Biol Control 6:412–421

Heimpel GE, Lundgren JG (2000) Sex ratios of commercially reared biological control agents. Biol Control 19:77–93

Hoelmer KA, Kirk AA, Pickett CH, Daane KM, Johnson MW (2011) Prospects for improving biological control of olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae), with introduced parasitoids (Hymenoptera). Biocontrol Sci Technol 21:1005–1025

Holt R, Lawton J (1994) The ecological consequences of shared natural enemies. Annu Rev Ecol Syst 25:495–520

Jackson CG, Long JP, Klungness LM (2008) Depth of pupation in four species of fruit flies (Diptera: Tephritidae) in sand with and without moisture. J Econ Entomol 91:138–142

Johnson MW, Daane KM, Wang X-G, Yokoyama V, Pickett CH, Hoelmer KA, Kirk AA (2012) Biological control of olive fruit fly. Integr Prot Olive Crops IOBC/WPRS Bull 79:63–69

Jones TW (1982) Sex ratio and host size in parasitoid wasp. Behav Ecol Sociobiol 10:207–210

Kenneth W, Hardy ICW (2002) Statistical analysis of sex ratios: an introduction. In: Hardy ICW (ed) Sex ratios concepts and research methods. Cambridge University Press, Cambridge, pp. 48–92

King BH (1988) Sex ratio manipulation in response to host size by the parasitoid wasp *Spalangia cameroni*: a laboratory study. Evolution 42:1190–1198

Liquido NJ, Shinoda LA, Cunningham RT (1991) Host plants of the Mediterranean fruit fly (Diptera: Tephritidae): an annotated world review. Misc Publ Entomol Soc Am 77:1–52

Malheiro R, Casal S, Baptista P, Pereira JA (2015) A review of *Bactrocera oleae* (Rossi) impact in olive products: from the tree to the table. Trends Food Sci Technol 44:226–242

Martín-Vega D, Hall MJR, Simonsen TJ (2016) Resolving confusion in the use of concepts and terminology in intrapuparial development studies of Cyclorrhaphous Diptera. J Med Entomol 53:1249–1251

Napoleon ME, King BH (1999) Offspring sex ratio response to host size in the parasitoid wasp *Spalangia endius*. Behav Ecol Sociobiol 46:325–332

Nugnes F, Bernardo U, Viggiani G (2017) An integrative approach to species discrimination in the *Anagrus atomus* group *sensu stricto* (Hymenoptera: Mymaridae), with a description of a new species. Syst Biodivers 15:582–599

Nugnes F, Russo E, Viggiani G, Bernardo U (2018) First record of an invasive fruit fly belonging to *Bactrocera dorsalis* complex (Diptera: Tephritidae) in Europe. Insects 9:182

Pašková M (2007) New larval agar-based diet for laboratory rearing of Mediterranean fruit fly *Ceratitis capitata* (Diptera, Tephritidae). Biol Bratisl 62:477–481

R Core Team (2018) R: a language and environment for statistical computing. R Foundation for statistical computing, Vienna, Austria. http://www.r-project.org

Rice RE, Phillips PA, Stewart-Leslie J, Sibbett GS (2003) Olive fruit fly populations measured in central and southern California. Calif Agric 57:122–127

Shelly T, Epsky N, Jang EB, Reyes-Flores J, Vargas R (2014) Trapping and the detection, control, and regulation of tephritid fruit flies. Springer, New York

Silvestri F (1913) Viaggio in Africa per cercare parassiti di mosche dei frutti. Boll Lab Zool Gen Agrar R Portici 8:3–164

Silvestri F (1915) Descrizione di nuovi imenotteri Calcididi africani. Boll Lab Zool Gen Agrar Portici 9:337–377

Statgraphics plus version 3.0. (1997) Rockville (MD): Manugistics

Storck V, Karpouzas DG, Martin-Laurent F (2017) Science of the total environment. Sci Total Environ 575:1027–1033

Szyniszewska AM, Tatem AJ (2014) Global assessment of seasonal potential distribution of Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). PLoS ONE 9(11):e111582

Viggiani G, Bernardo U, Sasso R (2007) Description of *Baryscapus silvestrii*, n. sp. (Hymenoptera: Eulophidae), a new gregarious parasitoid of the olive fly *Bactrocera oleae* (Gmelin) (Diptera: Tephritidae) in southern Italy. Boll Lab Ent Agrar F Silvestri 61:63–70

Vinson S (1976) Host selection by insect parasitoids. Annu Rev Entomol 21:109–133

Zucchi RA (2001) Mosca-do-Mediterrâneo, *Ceratitis capitata* (Diptera, Tephritidae). In: Vilela EF, Zucchi RA, Cantor F (eds) Histórico e impacto das pragas introduzidas no Brasil. Holos, Ribeirão Preto, pp 15–22

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