SÃO PAULO STATE UNIVERSITY - UNESP

JABOTICABAL CAMPUS

HOST REGULATION-RELATED PEPTIDES FROM THE ENDOPARASITOID *Cotesia flavipes* (HYMENOPTERA: BRACONIDAE): IDENTIFICATION, IMMUNOSSUPRESSIVE FUNCTIONS AND INSECTICIDAL POTENTIAL AGAINST *Diatraea saccharalis* (LEPIDOPTERA: CRAMBIDAE)

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“I think”

Charles Darwin

Ao Pedrinho, que está lá arriba...

Dedico

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PEPTÍDEOS RELACIONADOS À REGULAÇÃO HOSPEDEIRA DO ENDOPARASITOIDE *Cotesia flavipes* (HYMENOPTERA: BRACONIDAE): IDENTIFICAÇÃO, FUNÇÕES IMUNOSUPRESIVAS E POTENCIAL INSETICIDA EM *Diatraea saccharalis* (LEPIDOPTERA: CRAMBIDAE)

Resumo

A broca-da-cana, *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae), é uma das principais pragas da cultura da cana-de-açúcar e tem sido manejada com sucesso em determinadas regiões do Brasil com o uso do parasitoide *Cotesia flavipes* Cameron, 1891 (Hymenoptera: Braconidae). Vespas endoparasitoides como *C. flavipes* são capazes de regular a fisiologia de seus hospedeiros por meio da secreção de proteínas bioativas sintetizadas por glândulas de veneno, vírus simbiontes, teratócitos e pela própria larva em desenvolvimento na hemocele do hospedeiro. O veneno dos parasitoides é injetado para auxiliar o estabelecimento do parasitoide no interior do hospedeiro nos primeiros dias após a oviposição. Teratócitos são células extraembrionárias que se dissociam da serosa após a eclosão do parasitoide e se dispersam na hemocele do hospedeiro durante o desenvolvimento do parasitoide. A atividade dos teratócitos influencia diretamente a fisiologia do hospedeiro por meio da secreção de peptídeos específicos. Muitos peptídeos do veneno e dos teratócitos de parasitoides foram identificados, mas muitos são não caracterizados e explorados. O objetivo deste estudo foi aplicar técnicas modernas de proteo-transcritômica para a identificação de proteínas produzidas durante o parasitismo de *C. flavipes* sobre *D. saccharalis* com destaque para proteínas produzidas pelos teratócitos e glândulas de veneno do parasitoide. Uma grande gama de proteínas e peptídeos expressos pelos teratócitos e glândula de veneno de *C. flavipes* foi identificada. Em seguida, alguns desses peptídeos foram selecionados e avaliados em termos imunossupressivos e inseticida sobre lagartas de *D. saccharalis*. Os resultados obtidos indicaram funções imunossupressoras e biotecnológicas úteis para auxiliar a compreensão da fisiologia do parasitismo bem como para o desenvolvimento de novas moléculas inseticidas.

Palavras-chave: controle biológico, proteômica, biotecnologia, transcritômica, fisiologia do parasitismo.

HOST REGULATION-RELATED PEPTIDES FROM THE ENDOPARASITOID *Cotesia flavipes* (HYMENOPTERA: BRACONIDAE): IDENTIFICATION, IMMUNOSSUPRESSIVE FUNCTIONS AND INSECTICIDAL POTENTIAL AGAINST *Diatraea saccharalis* (LEPIDOPTERA: CRAMBIDAE)

Abstract

The sugarcane borer, *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae), is one of the most relevant pests in sugarcane fields and has been successfully managed in certain regions of Brazil with the use of the parasitoid *Cotesia flavipes* Cameron, 1891 (Hymenoptera: Braconidae). Endoparasitoids regulate the behavior and physiology of their hosts by secreting bioactive proteins synthesized by venom glands, symbiotic viruses, teratocytes or by the developing larva in the host's hemocoel. Venom is injected into the host to aid parasitoid successful establishment in the first days of parasitism. Teratocytes are extraembryonic cells present in the parasitoid egg that dissociate from the serosa after the parasitoid hatches and disperse in the host insect's hemocoel. Several venom- and teratocytes-derived peptides have been identified in different parasitoid wasps and many remain uncharacterized or unexplored. Investigations of the identity of host regulation-related molecules from *C. flavipes* may result in the identification of proteins with biotechnological potential. The purpose of this research was to apply modern proteotranscriptomic approaches for the identification host regulation-related proteins from *C. flavipes*, highlighting peptides from teratocytes and venom. A plenty of peptides produced by teratocytes and venom glands was identified. Following, some of these peptides were selected and evaluated in terms of immunosuppressive and insecticidal potentials against *D. saccharalis* larvae. Results point out biological and biotechnological potential of these peptides that shed light on the comprehension of the physiology of parasitism as well for the development of new insecticidal molecules.

Keywords: biological control, proteomics, biotechnology, transcriptomics, parasitism physiology.

Chapter I - Overview

1. Introduction

After parasitism, koinobiont parasitoids allow the movement and uptake of nutrients by their hosts, but, like other parasitoids, usually kill their hosts at the end of the parasitic larval stage. For successful development, parasitoids need to alter the physiology and behavior of their hosts, creating a favorable environment for parasitoid immature development. This ability of parasitoids is so-called host regulation, which can be classified in four main mechanisms of maternal or embryonic origin (Strand, 2014, Ali et al. 2015). The first maternal mechanism is the injection of venom or calix fluid by the female parasitoid wasp in the host along with the eggs during oviposition (Asgari and Rivers 2011; Salvia et al. 2021). The second maternal mechanism is the injection of symbiotic viruses, also injected into the host during oviposition (Gundersen-Rindal et al. 2013). Finally, there are 2 host regulation mechanisms of embryonic and post-embryonic origin, which are related, respectively, with the secretion of host regulation-related molecules by teratocytes and the developing parasitoid larvae (Dahlman 1990, Strand 2014).

Teratocytes are cells formed during the embryonic development of the parasitoid and come from the dissociation of the serosa just after hatching of the parasitoid larva. Serosa is a layer of extraembryonic cells that surrounds the embryo (Strand 2014). In general, after dissociation in the host's hemolymph, teratocytes develop independently and are distributed all over the hemocoel of the host (Dahlman 1991). In addition to the substantial increase in size, these cells show few external morphological changes during the development period of the parasitoid larvae (Hotta et al. 2001, Gao et al. 2016).

This type of cell can alter various physiological functions of the hosts for the successful development of the parasitoid larva. Among these host regulation functions, some are highlighted, such as immunosuppression (Rana et al. 2002; Ali et al. 2015), control of the endocrine system (Pennacciho et al. 1994), regulation of nutrition (Cônsoli et al. 2001), the control of protein synthesis (Kadono-Okuda et al. 1998), metamorphosis anomalies (Shi et al., 2015), neuropeptide reprogramming (Shi et al., 2016), enzymatic

degradation of fatty body (Nakamatsu et al. 2002), among other physiological changes (Rana et al. 2002; Caccia et al., 2012).

Structurally, teratocytes studied to date are polyploid cells, hypertrophied, opaque, spherical and possible to be visualized by naked eye (Shelby et al. 2014). The cellular structure of braconid teratocytes contains a plasma membrane composed of a dense layer of microvilli (Zhang et al. 1994). Moreover, teratocytes present cellular organelles such as rough endoplasmic reticulum and mitochondria close to the plasma membrane and a large Golgi complex. This cellular structure suggests a high metabolic activity suitable both for nutrients acquisition and for the synthesis and secretion of bioactive proteins to aid larval parasitoid development (Vinson and Scott 1974; Zhang et al. 1994).

Transcriptomic analysis of the teratocytes from *Cotesia pluttelae* (Kurdjumov, 1912) (Hymenoptera: Braconidae) indicated the presence of transcripts possibly associated with the manipulation of the host's physiology such as transcripts associated with insulin signaling and biosynthesis of important hormones, in addition to digestive enzymes, serpins and GTPase activators (Ali et al. 2015). Part of the physiological changes in parasitized caterpillars is associated with changes in the functioning of their endocrine systems, due, for example, to the reduction of expression of essential esterases for the degradation of juvenile hormone and the interruption of the expression of ecdysteroid receptors in the host (Ali et al., 2013).

A great part of the hymenopteran parasitoids of the Braconidae family are koinobionts endoparasitoids. In this kind of interaction, teratocytes are also related with the production of antimicrobial compounds to maintain the sanity in the host's hemocoel, since the host is immunosuppressed after parasitism. For example, *Cotesia vestalis* (Haliday, 1834) (Hymenoptera: Braconidae) secretes antimicrobial peptides that protect the host *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae) against entomopathogenic bacteria, consequently protecting the parasitoid located inside the host (Gao et al. 2016). Additionally, an increased activity of lysozyme was observed in larvae of *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae) parasitized by *Cotesia flavipes* Cameron, 1891 (Hymenoptera: Braconidae) (Pinto et al. 2019).

Little information regarding the molecular composition of *C. flavipes* teratocytes parasitizing *D. saccharalis* is available. So far, it is known that *C. flavipes* teratocytes are

responsible for the expression of a transcript that encodes a serpin and a transcript that encodes a protein that inhibits protein translation in the host (Rossi 2012).

In addition to the knowledge of the identity and the unraveling of the biological activity of the molecules involved in regulating the physiology of the host, these molecules derived from parasitoids can also be exploited for the control of insect pests. For example, transgenic plants containing genes from parasitoids are able to cause adverse effects in insect pests after ingestion (Maiti et al. 2003; Fath-Goodin et al. 2006, Rossi et al. 2012; Di Lelio et al. 2014, Kim et al. 2016; Wei et al. 2016a, Wei et al. 2016b, Merlin et al. 2020).

In the case of transgenic events in sugarcane for pest control, most information is still limited to academic research or preliminary tests. There are two transgenic sugarcane events commercially available in Brazil with resistance to insects (CTNBio, 2021). These events present, individually, the gene *cry1Ab* or *cry1Ac*, also present in commercial varieties of corn and cotton, and confers resistance to the sugarcane borer *D. saccharalis*. Even with the emergence of this new technology, resistance of target and non-target pests to the protein Cry1Ab has already been reported (Ghimire et al. 2011, Bernardi et al. 2011, Omoto et al. 2016). In addition, in Argentina, the sudden appearance of *D. saccharalis* resistant to the MON 89034 × MON 88017 corn event was reported, which expresses the proteins Cry1A.105 and Cry2Ab2 (Grimi et al., 2018). Such evidence shows that new transgenic events launched on the market may be compromised by the pre-existence of resistant pests in related crops due to the limited variety of modes of action of insecticidal proteins commercially exploited today. These observations make the demand by new active principles with different modes of action a continuous task.

There is a great diversity of parasitoid hymenopterans in nature, and even considering only those species belonging to *C. flavipes* complex (Muirhead et al. 2008), it is remarkable that this group of insects is still a practically unexplored source of molecules with biotechnological potential (Beckage and Gelman, 2004, Rossi et al. 2012). For the identification of this biotechnologically relevant molecules, with emphasis in proteins or peptides, liquid chromatography has been applied in proteomics for the analysis of complex mixtures of proteins. The application of this method in samples collected from different tissues, and developmental stages makes possible the discovery of new proteins, often hidden when genomic-based approaches are applied (Huang et

al., 2012). In this regard, studies have been carried out with parasitoid hymenopterans to identify and characterize proteins with medical or agricultural applicability (Yan et al., 2016).

New insecticidal proteins derived from *C. flavipes* teratocytes or venom glands with different modes of action than that observed for Bt toxins are desirable, mainly thinking on strategies to delay the evolution of resistance in insect pest populations. These new proteins/peptides from *C. flavipes* may be expressed in new transgenic plants or pyramided with known commercial protein toxins to provide protection to plants of economic interest against insect pests.

Our aim was to perform biochemical and proteotranscriptomic studies to understand and identify the composition of proteins associated with host regulation of *C. flavipes* through *D. saccharalis* to understand biological functions in parasitism and evaluate the insecticidal potential of these molecules.

2. Literature Review

2.1. Parasitoid wasps

Parasitoids are insects that use the body of a host as a physical and nutritional substrate during larval stage, feeding on only one host to complete their parasitic stage. Although Hymenoptera and Diptera are the main orders with known parasitoid species, Lepidoptera, Coleoptera and Neuroptera also have parasitoid species described (Mills, 2009). Parasitoidea is a hymenopteran monophyletic group inside the suborder Apocrita and is characterized by the parasitoid lifestyle (Peters et al., 2017). This group has developed several strategies of host regulation. Among those strategies, briefly we may highlight the injection of venom toxins, teratocytes release, and injection of polydnavirus and virus-like particles that colonize host cells to produce molecules with physiological regulatory functions (Pennacchio et al. 2012, Strand 2014, Moreau 2015, Gauthier et al. 2018). A main host regulation function that is regulated by parasitoids is the humoral and cellular immune disruption, especially for protecting the parasitoid embryos (Falabella, 2018).

Different parasitoid guilds have been recorded and described according to their lifestyle and behavior (Mills 1992, Mills 1994, Mills 2009). All these diverse guilds have

an unexplored potential in agriculture, especially because thousands of parasitoid species remain undescribed (Jones et al. 2009). Parasitoid guilds can be classified according to, for example, offspring number per host, how they kill their host and the stage that their hosts are parasitized

2.2. Egg parasitoids

Egg parasitoids are those that parasitize their hosts in the egg stage. In Agriculture, the greatest advantage of using egg parasitoids is killing their hosts before any significant damage to the crop plants. Most known egg parasitoids are endoparasitoids idiobionts, those that interrupt the development of their hosts. However, some egg parasitoids such as *Chelonus inanitus* (Linnaeus, 1767) (Hymenoptera: Braconidae) are considered as multiple stage parasitoids because they allow the development of the host from egg to larva, being considered as egg-larval parasitoid.

In Brazil, the use of the egg parasitoid *Trichogramma galloi* Zucchi, 1988 (Hymenoptera: Trichogrammatidae) for the management of the sugarcane borer increased from 6 to 22 percent of the total sugarcane area in only 5 years, totalizing an area of two million ha (Parra 2014, Parra and Coelho 2019). Due to its efficacy in Brazil, *T. galloi* is commercialized as a biological control agent, with seven registered products for use in agriculture (Table 1).

For a successful implementation of a biological control program using egg parasitoids, it is necessary to consider the preference of the parasitoid for the embryonic phase in which the parasitism is performed. Parasitoid oviposition preference varies among host species and lineages (Tian et al. 2017). Egg parasitoids analyze the host suitability by touching the egg surface several times with the antennas. This behavior known as drumming aids host selection (Du et al. 2018). Some egg parasitoids prefer hosts at the initial phase of embryonic development while others prefer hosts in more advanced embryonic stages, but there are parasitoids with no preference (Pastori et al. 2010, Hill et al. 2019).

Egg parasitoids, such as those from the Platygastridae family, are relevant biological control agents, occurring naturally on *Megacopta cribraria* (Fabricius, 1798) (Hemiptera: Plataspidae), an important soybean pest in the U.S.A (Knight et al. 2017).

Natural occurrence of scelionids, such as *Telenomus triptus* Nixon (Hymenoptera: Scelionidae) *and Telenomus cyrus* Nixon, 1937 (Hymenoptera: Scelionidae) in Asia and *Telenomus podisi* Ashmead, 1893 (Hymenoptera: Scelionidae) in southern Brazil have been also reported (Idalgo et al. 2013, Todoroki et al. 2015, Srikumar et al. 2015). In Brazilian soybean fields, 80 percent of naturally parasitized pentatomid eggs are from the insect pest *Euschistus heros* (Fabricius, 1798) (Hemiptera: Pentatomidae) (Aquino et al. 2019). Similar results are reported for eggs from the insect pest *Tibraca limbativentris* Stål, 1860 (Hemiptera: Pentatomidae) in irrigated rice fields (Riffel et al. 2010).

2.3. Larval parasitoids

Larval parasitoids are also widely used in biological control programs. *Cotesia* spp. parasitoids represent the fourth most used taxon in augmentative biological control, especially in South America and China (van Lenteren et al. 2018). The use of larval endoparasitoids has been widely discussed in fruticulture but such strategy is still poorly commercially explored. Even though, several larval parasitoids occur naturally parasitizing fruit flies (Tephritidae), especially larval-pupal koinobiont endoparasitoids (Paranhos et al. 2019).

Braconids prefer to parasitize fruit flies (Tephritidae) larvae at the third instar, however parasitism may occur at the first and second instar in lower rates (Ledezma et al. 2013). This preference is related to the searching ability of females, once parasitoids easily detect larger hosts inside the fruits (Ovruski 1994). For example, the braconid *Diachasmimorpha longicaudata* (Ashmead, 1905) (Hymenoptera: Braconidae) prefers to parasitize third instar larvae of *Anastrepha fraterculus* (Wiedemann, 1830) (Diptera: Tephritidae) generating a larger number of decedents (van Nieuwenhove and Ovruski 2011, López et al. 2009). In this case, a larger host provides better nutritional conditions to meet the requirements for parasitoid offspring development.

For a successful applied biological control with parasitoid wasps, mass rearing must be efficient and uninterrupted, lasting several generations in the same host species. For example, the pupal parasitoid *Psyttalia concolor* Szepligeti, 1910 (Hymenoptera: Braconidae) has been successfully reared for several generations using *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae) larvae as hosts without any changes in the

efficiency of parasitism (Giunti 2016). Nevertheless, it should be kept in mind that ingredients of the diet in which the hosts are reared have a direct influence in the quality of the parasitoid (Ongaratto et al. 2019).

In terms of treated area, the most successful example of an applied biological control program using larval parasitoids is the use of the larval endoparasitoid *Cotesia flavipes* Cameron, 1891 (Hymenoptera: Braconidae) for management of the sugarcane borer *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae). This biological control program started in the 1970s in Brazil and nowadays has been applied in 40 percent of the Brazilian sugarcane fields (Parra 2014, Parra and Coelho 2019). The parasitoid *C. flavipes* is approved for commercialization in Brazil, which stimulates the replacement of chemical products by this biological control agent (Table 1).

Table 1. Commercially available parasitoids approved for use in Brazil (http://agrofit.agricultura.gov.br; MAPA 2020).

Biological control agent	No. products	Target pests	Crops
Cotesia flavipes	27	*Diatraea saccharalis*	All
Trichogramma galloi	7	*Diatraea saccharalis*	All
Trichogramma pretiosum	8	*Tuta absoluta, Anticarsia gemmatalis, Spodoptera frugiperda, Helicoverpa zea*	All
Trissolcus basalis	1	*Nezara viridula*	All

2.4. Pupal parasitoids

Pupal parasitoids are important biological control agents for pest management in eucalyptus agroecosystems (Pereira et al. 2011, Chen et al. 2018). *Palmistichus elaeisis* Delvare & LaSalle, 1993 (Hymenoptera: Eulophidae) is a generalist pupal endoparasitoid with high potential for biological control of lepidopterans in eucalyptus (Pereira et al. 2009). This parasitoid was observed parasitizing pupae of *Thyrinteina arnobia* (Stoll, 1782) (Lepidoptera: Geometridae), *Thyrinteina leucoceraea* Rindge, 1961 (Lepidoptera: Geometridae), species of the genus *Hylesia* Hübner, 1820 (Lepidoptera: Saturniidae) and others (Pereira et al. 2008, Pereira et al. 2009). However, lepidopterans have several defense mechanisms against parasitoid attacks. For example, during parasitism by *P.*

elaesis, pupae of *T. arnobiae* and *Hylesia sp*. start rotational abdominal movements that expel the parasitoid, failing the oviposition (Soares et al. 2009).

2.5. Adult parasitoids

The parasitoid *Aphidius colemani* Viereck, 1912 (Hymenoptera: Aphidiidae) is an aggressive control agent for adult aphids in diverse plant species. This parasitoid is resistant to methoxyfenozide and indoxacarb, which is a valuable feature in Integrated Pest Management (IPM) (Stara et al. 2011). Plant fertilization may aid parasitoid establishment in agroecosystems, since it was observed that adult emergence and longevity of the parasitoid *Aphidius rhopalosiphi* de Stefani-Perez, 1902 (Hymenoptera: Aphidiidae) is positively influenced by nitrogen fertilization of plants in which the host *Sitobion avenae* (Fabricius, 1775) (Hemiptera: Aphidae) is feeding (Aqueel et al. 2015). On the other hand, nitrogen fertilization may present a positive influence on the development of phytophagous insects (Clemensen et al. 2017, Pinto and Ongaratto 2019). Thus, in terms of IPM and biological control, nitrogen fertilizers must be carefully managed to avoid pest insurgence.

Parasitoids of coleopterans may be relevant for controlling important pests like curculionids. An important case of success has been recently described for controlling the South American weevil *Listronotus bonariensis* (Kuschel, 1955) (Coleoptera: Curculionidae) with the parasitoid *Microctonus hyperodae* Loan, 1974 (Hymenoptera: Braconidae) in New Zealand (Barker 2013). The parasitoid was release into New Zealand in 1991 and successfully established in the new environment after reproductive adaptations.

3. Host regulation tools in parasitoids

Parasitoid wasps, especially those from the Braconidae and Ichneumonidae families, can change the physiology and behavior of their hosts using diverse host regulation mechanisms (Glupov and Kryukova 2016). These regulations aid, for example, parasitoids to overcome their hosts' immune systems and create a favorable environment for parasitoid immature development. This regulatory phenomenon is so-called host

regulation and the origin of the factors involved in this process can be classified as maternal, embryonic or post-embryonic (Strand 2014, Ali et al. 2015).

3.1. Factors from maternal origin

Factors of maternal origin are those produced in parasitoid female wasps that are deposited along with the eggs in the host that aid parasitoid development. As examples of factors from maternal origin, there are calix fluids from the ovary (Salvia 2021), virus-like particles (VLPs) (Pennacchio and Strand 2006), symbiotic polydnaviruses (PDVs) (Strand and Burke 2013) and venom (Asgari et al. 2003). These factors are responsible for changes in the host such as modulation of genes expression (Chevignon et al. 2015), alter in the reproductive and foraging behaviors (Mathé-Hubert et al. 2016), halting host development, and inhibition of the immune system (Mrinalini and Werren 2017).

3.2. Embryonic-derived factors

The mechanisms of host regulation of embryonic origin are related to the host regulatory activity promoted by teratocytes (Strand 2014, Glupov and Kryukova 2016). Teratocytes are polyploid cells formed during the embryonic development of the parasitoid and are dissociated from the serosa, a layer of cells that surrounds the embryo, after hatching of the parasitoid larva inside the hosts (Dahlman 1990, Strand 2014) (Fig. 1).

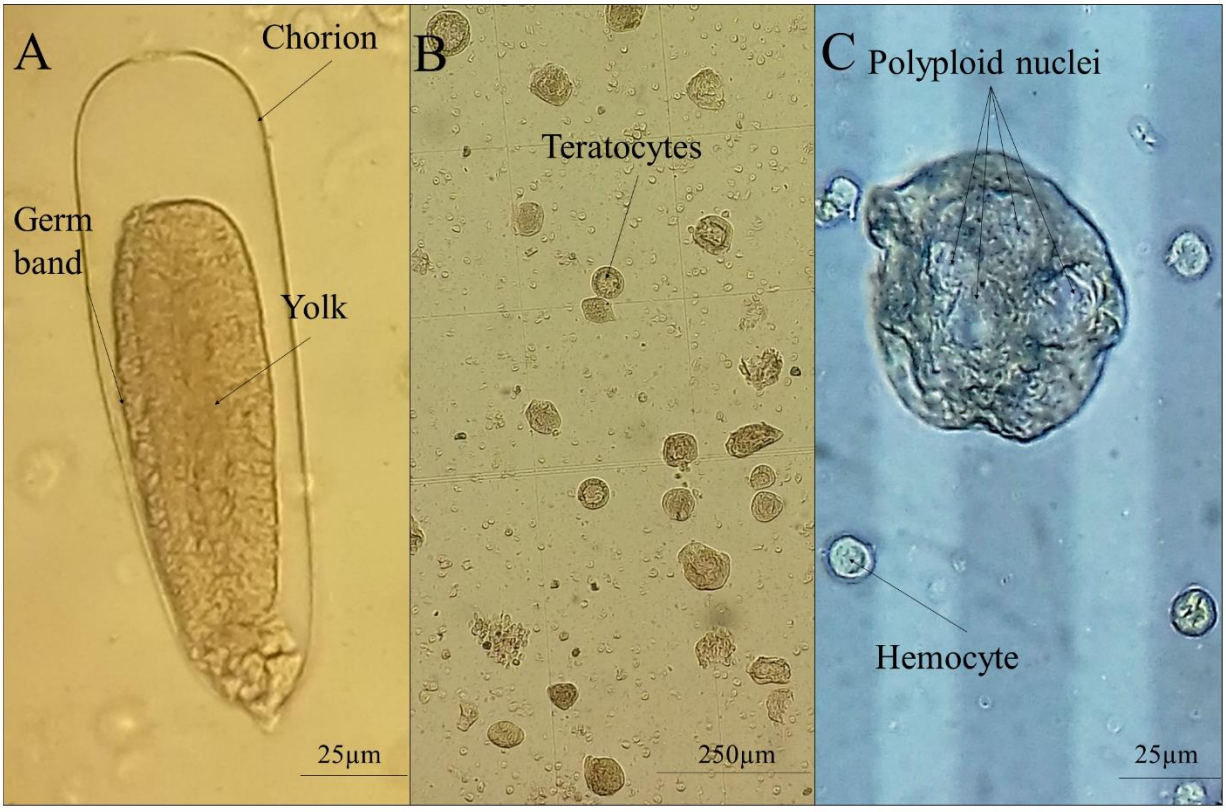

Figure. 1. Origin and overall structure of *Cotesia flavipes* teratocytes. A- *C. flavipes* egg with arrows pointing the location of the germ band, egg chorion and yolk (cell layer from which teratocytes dissociate), is clearly differentiated. **B**- Overview of teratocytes in the host hemolymph. **C**- Zoom view of *C. flavipes* teratocytes surface showing its polyploid nuclei and size comparison with the hemocytes of the host *Diatraea saccharalis*.

Teratocytes are known for modulating the host immune system (Ali et al. 2015; Gao et al. 2016), endocrine system (Pennacciho et al. 1994), host nutrition (Cônsoli et al. 2001), protein synthesis (Kadono-Okuda et al. 1998), metamorphosis abnormalities (Shi et al. 2015), enzymatic degradation of the fatty body (Nakamatsu et al. 2002), among others (Rana et al. 2002, Caccia et al. 2012). Despite extensive knowledge about the functions of teratocytes, there is a limited knowledge about the molecules associated with the regulatory functions of this cell type.

3.3. Post-Embryonic-derived factors

Parasitoid larvae, in addition to feeding on their host's body fluids, also play an important regulatory role the physiology of the host. For example, endoparasitoid larvae

of the genus *Glyptapanteles* Ashmead, 1904 inhibits enzymes related to their host's hormonal activities and alter host behavior (Schafellner et al. 2007). In addition, parasitoid larvae produce protein secretions that directly interfere with the host's immune system by inhibiting phenoloxidases and reducing the activity of hemocytes (Bischof and Ortel 1996, Hoch et al. 2002, Hartzer et al. 2005). The saliva of parasitoid larvae also plays an important role in suppressing the immunity of their hosts using protein factors that inactivate hemocytes or reduce the activation of phenoloxidase (Richards 2012).

4. Potential use of host regulation-related molecules in agriculture

Due to the wide diversity of parasitic factors that act during the host regulation, parasitoids are considered a large reservoir of biomolecules with biotechnological potential that can be used in the control of insect pests (Di Lelio et al. 2014). For example, a cystatin gene from the symbiotic polydnavirus of *Cotesia vestalis* (Haliday, 1834) (Hymenoptera: Braconidae) was inserted in a transgenic tobacco plant. Larvae of *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae) fed with the transgenic plants presented high mortality in their first instars. Potential effects of these transformed tobacco plants were also observed against other insect pests such as *Helicoverpa assulta* (Guenée, 1852) (Lepidoptera: Noctuidae) and *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae) (Kim et al. 2016). Another gene from the symbiotic polydnavirus of *Cotesia rubecula* (Marshall, 1885) (Hymenoptera: Braconidae) (CrV1) was inserted in a Baculovirus (AcMNPV-CrV1). The recombinant entomopathogen presented increased insecticidal activity over larva of *Pieris rapae* (Linnaeus, 1758) (Lepidoptera: Pieridae) (Wei et al. 2016).

Teratocytes-derived molecules has shown to have an interesting potential for pest control via plant transgeny. The first studied indicated a higher mortality of the pests *Chloridea virescens* (Fabricius, 1777) (Lepidoptera: Noctuidae) and *Manduca sexta* (Linnaeus, 1763) (Lepidoptera: Sphingidae) ingesting transgenic tobacco plants expressing TPS14, a teratocyte protein from the parasitoid *Microplitis croceipes* (Cresson, 1872*)* (Hymenoptera: Braconidae). Other studies also showed the insecticidal potential for several pest species of a chitolectin (*TcChit*) from the teratocytes of the

parasitoid *Toxoneuron nigriceps* (Viereck) (Hymenoptera: Braconidae) inserted into tobacco and solanum plants (Rossi et al. 2012, Merlin et al. 2020) (Table 2).

Table 2- Genetically modified organisms with genes derived from parasitoids and adverse effects of the heterologous peptide on insect pests.

Parasitoid	Target pest	Regulation factor	Toxin	Effect	Reference
Microplitis croceipes	*Chloridea virescens; Manduca sexta*	Teratocytes	TPS14	Slow growth rate and increase of mortality	Maiti et al. (2003)
Compoletis sonorensis	*Heliothis virescens*	Polydnavirus	AHv1.0, A'Hv0.8, VHV1.1, VHv1.4 e WHv1.6	Inhibition of growing, lower foliar consumption and increase of mortality	Fath-Goodin et al. (2006)
Toxoneuron nigriceps	*Chloridea virescens*	Teratocytes	Tnchi	Higher larval mortality	Rossi et al. (2012)
Toxoneuron nigriceps	*Spodoptera littoralis*	Polydnavirus	TnBVANK1	Inhibition of growing and increase of mortality	Di Lelio et al. (2014)
Cotesia plutellae	*Spodoptera exigua*	Polydnavirus	CpBV-CST1	Higher larval mortality	Kim et al. (2016)
Cotesia rubecula	*Pieris rapae; Spodoptera exigua*	Polydnavirus	CVr1	Higher larval mortality	Wei et al. (2016a); Wei et al. (2016b)*
Toxoneuron nigriceps	*Chrysodeixis includens; Spodoptera albula; Spodoptera frugiperda; Tuta absoluta; Bemisia tabaci*	Teratocytes	TcChit (Tnchi)	Lower mortality; Lower probability of molting; Lower larval survival and pupal weight; Reduced adult emergence	Merlin et al. (2020)

*Study performed after transforming an entomopathogenic virus.

Parasitoid venom peptides have been least exploited for agricultural purposes but a vast knowledge about its composition is available. A recent study revealed that the venom of the endoparasitoids wasp *Aenasius arizonensis* (Girault, 1915) (Hymenoptera: Encyrtidae) present insecticidal activity against its host *Phenacoccus solenopsis* Tinsley, 1898 (Hemiptera: Pseudococcidae) (Abbas, 2021). Furthermore, an endonuclease from the parasitoid wasp *Pteromalus puparum* (Linnaeus, 1758) (Hymenoptera: Pteromalidae) induced cellular death in lepidopteran cell lines, being considered for the authors a potential insecticidal peptide with known mode of action (Wang et al. 2020).

Host regulation molecules are potentially more selective (active against the host and innocuous to non-target organisms) that those insecticidal molecules currently used in Integrate Pest Management (IPM), and molecules from parasitoids can also serve as a basis for the rational design of molecules with new modes of action. For this, more researches need to be carried out to elucidate efficiency and the mechanisms of action involved in host regulation, as well studies to improve the insecticidal power of these molecules.

5. Cotesia flavipes and Diatraea saccharalis

The parasitoid *Cotesia flavipes* Cameron, 1891 (Hymenoptera: Braconidae) is native from Indo-Australian region and has been widely introduced in the Neotropical region and Asia for the management of insect pests. This species was selected for introduction in the Americas due to its successful history of parasitism in stem borers in Australia (Overholt et al. 1994).

In its center of origin, *C. flavipes* has been reported to parasitize different grass borers, in addition to other lepidopterans (Overholt et al. 1997). In the neotropical region, *C. flavipes* attacks several lepidopterans of the genus *Diatraea* Guilding, 1828 and present great importance for insect pest management, such as the sugarcane borer *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera).

In Brazil, the parasitoid *C. flavipes* is used as the main biological control agent for *D. saccharalis* due to its control efficiency. The introduction of this species in Brazil was made using insects from Trinidad-Tobago and took place in two stages, one in the southeast and the other in the northeast of Brazil in the years 1971 and 1974, respectively. In 1978, new strains of *C. flavipes* from India and Pakistan, cooler and wetter regions, were introduced in the São Paulo state (Macedo 1978). The introduction of this natural enemy reduced the infestation of the sugarcane borer from 11% to 2.8% between 1980 and 2002 (Polanczyk et al. 2004).

In the last decade (2010's), *C. flavipes* was mass reared and released to control larval *D. saccharalis* in approximately 40% of the whole sugarcane area of Brazil (Parra, 2014). Currently (2020's), it reaches more than ~4 million ha, representing ~66% of total sugarcane area (personal communication). Being a gregarious parasitoid, a single

oviposition of *C. flavipes* into *D. saccharalis* (Fig. 2) may generate more than 50 new parasitoids in one single host.

Figure 2. The endoparasitoid wasp *Cotesia flavipes* parasitizing the sugarcane borer *Diatraea saccharalis*.

6. Proteo-transcriptomics for exploring host regulation-related peptides

The first step for studying an insect venom is unraveling its components. The term “venomics” was first used in a proteomic study about snake venom composition (Juarez et al. 2004). Subsequently, venomics started to be applied for genomic and proteomic studies of venom (Menez et al. 2006). Currently, it has been used to describe studies in all, including combined, levels of central dogma of molecular biology (Sunagar et al. 2016).

Toxin-profiling studies, or “venomics”, of several species of parasitoid wasps have been performed using distinguished molecular tools, mainly transcriptomics and proteomics or both. Parasitoid venom toxins vary according species, but, in general,

parasitoid venom are composed by peptides related to immune suppression, cellular disfunction, and the presence of neuropeptides and antimicrobial peptides (Yokoi et al. 2017, Qi et al. 2015, Liu et al. 2018, Vincent et al. 2010, Sim and Wheeler 2016, Becchimanzi et al. 2020). Proteome analysis for predicting toxins must be included to genome or transcriptome data to make the investigation reliable because isolated proteome-based data may increase the number of false positives and therefore overestimates putative toxin matches (von Reumont et al. 2018, von Reumont et al. 2020).

The integration of transcriptomics and proteomics aiming the identification of the venom components of *C. flavipes* responsible for the host regulation effects observed in the laboratory over the host *D. saccharalis* was never previously performed. In this work we investigated such interaction with multiple biochemical and molecular tools, including immune competence and neonate mortality after ingestion of selected peptides from the parasitoid. The present study sheds light on the identity of host regulation molecules from venom and teratocytes of *C. flavipes*.

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Chapter II - First evidence for insecticidal potential of compounds produced during the parasitic phase of the endoparasitoid *Cotesia flavipes* (Hymenoptera: Braconidae)

Abstract

The immature stage of endoparasitoids develop inside their hosts. Consequently, endoparasitoids need to circumvent the action of the immune system and reprogram the physiology of their hosts to develop successfully. For this, parasitoids produce host regulation-related molecules that are secreted in the hemolymph of their hosts. These molecules come from the venom gland or the calix fluid of the female wasp, symbiotic viruses that infect host cells, teratocytes or from the larval parasitoid itself. However, the insecticidal potential of these molecules is not so often investigated. In here, we performed a survey for the biotechnological potential of compounds present in the hemolymph of the host *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae) parasitized by the endoparasitoid *Cotesia flavipes* Cameron, 1891 (Hymenoptera: Braconidae) as insecticides in feeding assays using *D. saccharalis* neonates as targets. Hemolymph serum from *D. saccharalis* larvae at 6 days after parasitism and hemolymph serum from non-parasitized *D. saccharalis* larvae at the same age were collected and incorporated into artificial diet or over sugarcane leaves and offered to *D. saccharalis* neonates as food. Following, hemolymph serum was fractioned using molecular filters and the fractions were tested for their insecticidal activity against *D. saccharalis* neonates. The use of sugarcane leaf disks as substrate for feeding assays indicated clear influences of hemolymph serum of parasitized hosts over *D. saccharalis* neonates than feeding assays using artificial diet. Hemolymph serum containing the contents of disrupted hemocytes and teratocytes from parasitized hosts presented a pronounced negative influence over the development of *D. saccharalis* neonates. Finally, hemolymph serum containing the content of disrupted cells was fractioned and the fractions containing proteins heavier than 100 kDa or between 3-10 kDa resulted in a reduction in leaf consumption by *D. saccharalis* neonates. There is an insecticide potential of hemolymph serum proteins of *D. saccharalis* larvae parasitized by *C. flavipes* and further investigations shall be performed aiming the identification of these molecules.

Keywords: sugarcane; protein fractions; insecticide discovery, parasitoid.

1. Introduction

The maximization of the use of agricultural areas for supplying the demand of food, energy and fibber production of the world population is constantly required. In order to improve productivity, it is necessary to optimize the use of exploited areas instead of exploring new ones (Oerke, 2006). Among other important actions, crops should be protected from adverse phytophagous insects, which are expected to be more harmful in a worming climate (Deutsch et al. 2018).

The sources of nowadays widely employed commercial insecticides are microorganisms or plants (Casida and Quistad 1998, Aguiar-Menezes 2005, Berdy 2005, Rattan 2010, Coloma et al. 2010, Miresmailli and Isman 2014). For instance, the insecticidal Cry and Vip proteins, widely used in transgenic plants, are derived from the microorganism *Bacillus thuringiensis* Berliner (Melo et al., 2016). Pyrethrins and nicotines, natural insecticides or precursor molecules of neurotoxic insecticides, are secondary compounds of *Chrysanthemum cinerariaefolium* Vis. (Asteraceae) and *Nicotiana* spp., respectively (Gajendiran and Abraham 2018, Zenkner et al. 2019).

Despite the efficacy of nowadays insect pest control tactics, there are issues regarding the toxicity and safety of use of such molecules or regarding their loss of efficacy given by the selection of resistant insect pests (Hawkins et al. 2019). Therefore, the discovery and development of ways to use new molecules with distinct modes of action to control insect pests are constantly demanded (Sudo et al. 2018).

In nature, there are still countless potential sources of molecules with different modes of actions, most untapped or restricted to academic studies. For example, arachnid toxins (King and Hardy 2013, Zhu et al. 2016, King, 2019), shell toxins (Gao et al. 2017), plant secondary compounds (Dang and van Damme 2015), parasitoid-derived proteins (Maiti et al. 2003, Rossi et al. 2012, Kim et al. 2016, Merlin et al. 2020) and several microorganisms (Fan et al. 2007, Melo et al. 2016, Shen et al. 2017). Among those, parasitoid proteins are the least exploited, even though being pointed as a rich source of bioactive molecules (Ali et al. 2015, Gao et al. 2016, Becchimanzi et al. 2020, Lin et al. 2019).

The successful development of hymenopteran parasitoids is given by their ability to alter the physiology and behaviour of their hosts, creating a favourable environment for

the immature development of their offspring. This characteristic of parasitoids is so-called host regulation, which can be classified as maternal or embryonic-derived mechanisms (Strand, 2014; Ali et al., 2015).

By releasing several host regulation molecules, parasitoids alter a number of host physiological functions that allows successful development of the endoparasitoid larva. Among these physiological regulation over their hosts, parasitoids alter immune competence (Rana et al. 2002, Ali et al. 2015), endocrine system (Pennacciho et al. 1994), host nutrition (Cônsoli et al. 2001, Salvador and Cônsoli 2008), protein synthesis (Kadono-Okuda et al. 1998), host metamorphosis (Shi et al. 2015), neuropeptide reprogramming (Shi et al. 2016), enzymatic degradation of host fat body (Nakamatsu et al. 2002), and other physiological changes in the host (Rana et al. 2002, Caccia et al. 2012, Merlin and Cônsoli 2019).

The parasitoid *Cotesia flavipes* Cameron, 1891 (Hymenoptera: Braconidae) is mass reared in Brazil and released in sugarcane fields for the management of the sugarcane borer, *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae) (Parra and Coelho 2019). Despite of the success in the use of *C. flavipes* as a biological control agent in sugarcane fields, we believe that *C. flavipes* may also contribute to insect pest management by providing host regulation related molecules with biotechnological potential to control insect pests, as seen for other parasitoids (Maiti et al., 2003; Rossi et al., 2012; Kim et al., 2016, Merlin et al. 2020). Thus, in this paper, we described a first survey of the viability of virulent molecules of *C. flavipes* as insecticides against *D. saccharalis*.

2. Material and Methods

2.1. Insect rearing, hemolymph extraction and sample preparation

Insects were obtained from a local laboratory (21°21′23″S, 48°3′48″W), São Paulo State, Brazil and were kept in controlled conditions during all developmental stages (25 ± 1°C; relative humidity 70 ± 10%; 12 h photo phase). Before parasitism over fifth instar *D. saccharalis*, parasitoids were kept in containers for 24 h to allow mating.

Each last instar *D. saccharalis* were parasitized by a single bout of one parasitoid. Control larvae at the same developmental stage were kept in the same conditions without

being parasitized. After each parasitism, the parasitoid was discarded. After 6 days, hemolymph samples were collected from parasitized and non-parasitized larvae by severing an anal false leg with an ophthalmic scissor. To avoid oxidation, the hemolymph samples were diluted (1:5; hemolymph: buffer) in cold anticoagulant buffer (0.098 M NaOH, 0.15 M NaCl, 0.017 M EDTA, 0.046 M citric acid) (Hotta et al. 2001).

Samples from non-parasitized (C) or parasitized (P) larvae were composed by extravasated hemolymph serum from non-parasitized larvae (Cs or Ps) or the hemolymph serum plus the cell contents (Cc or Pc). Hemolymph serums were collected by immediately centrifuging the hemolymph at low non-disruptive speed (4ºC; 5 min; 800 g). Supernatants were used as hemolymph serums. Hemolymph serums plus cell contents (Cc or Pc) were produced after cell disruption by one cycle of freezing and thawing followed by disruptive high-speed centrifugation (10 min x 7000 g) and pellet disruption with a pestle in Falcon-type tubes (15 mL). Subsequently, a final centrifugation (4°C; 5 min; 7000 g) was performed and the supernatant containing the hemolymph serum plus cellular soluble content was collected and cell debris was discarded. Finally, all samples were filtered through a 0.3 µm filter.

2.2. Bioassays in artificial diet

First, immature development of *D. saccharalis* was assessed in a long-term bioassay. Total protein titter was quantified using Bradford Reagent Commercial Reagent (Sigma®), following the manufacturer's recommendations (Bradford 1976). The bioassay was performed in flat-bottom cylindrical glass tubes (6 cm height ×2.5 cm diameter), containing 9.86 cm^3 of artificial diet. The surface of the artificial diet was covered with 600 µg of total protein for each protein source (Cs, Cc, Ps, Pc) and a control treatment containing only anticoagulant buffer. Twenty *D. saccharalis* neonates were placed in each tube. Five replicates (5 tubes containing 20 neonates) of each treatment were made. Larval survival (%), larval period (days), pupal weigh (mg), pupal period (days) and pupal survival (%) of *D. saccharalis* were observed.

2.3. Bioassays in sugarcane leaf disks

Sugarcane leaf discs (3.14 cm^2; IAC95-5000) were submerged in hemolymph samples (Cs, Cc, Ps, Pc) diluted in cold anticoagulant buffer (~6.66 µg $µL^{-1}$) and placed in 12-well cell culture flat plates (2.25 cm diameter) containing 1 mL of agar at the bottom (1%) to keep leaf humidity during bioassays. Subsequently, with a soft brush, twenty *D. saccharalis* neonate were placed on the leaf surface and the mortality evaluated after five days. Five replicates of each treatment were made. Each repetition was composed by one leaf disk containing twenty *D. saccharalis* neonates.

2.4. Fractioning and insecticidal potential of parasitoid secreted proteins in leaf discs

The contents of hemolymph plus cells contents from parasitized larvae (Pc) were fractioned using Amicom® molecular weight filters following the manufacturer instructions and Ashwin et al. (2018). There were obtained protein fractions of the following molecular weight (kDa) intervals: >100; 50-100; 30-50; 10-30; 3-10; <3.

Protein contents of the fractions were quantified using Bradford Reagent (Sigma-Aldrich®) and bovine serum albumin as standard (Bradford 1976), and samples were lyophilized, and resolubilized in deionized water at final protein concentration of 5 µg $µL^{-1}$. For the leaf disks bioassays, 40 µL of each protein fractions were applied over both leaf surfaces of the leaf disks using a brush. Leaf disks were allowed to superficially dry for five minutes and then were placed in a 12-well cell culture flat plate containing 1 mL of 1% agar at the bottom. Deionized water was used as control. We performed six replicates of each treatment. Each repetition was composed by one leaf disk containing twenty *D. saccharalis* neonates.

To access larval viability, larvae were touched with a soft brush and those that did not react were considered as dead. In addition, leaf consumption by larvae was assessed at three days after larvae were transferred to treated leaf disks. For this, leaf disks were collected, scanned and the area of the leaves consumed by neonates was measured using the software ImageJ v. 1.8.

2.5. Protein profiles visualization

Denaturing gel electrophoresis (SDS-PAGE) was performed to observe the protein profiles of samples Cc and Pc). Fractions with molecular weight ≥100 kDa were resolved in SDS-PAGE gels at 6.0% of polyacrylamide, fractions 50-100 kDa at 7.5%, and fractions 30-50, 10-30, 3-10 and ≤3 kDa at 12%. After resolved, gels were stained with Coomassie Brilliant Blue R250 Dye, following Laemmli (1970) and Sambrook and Russel (2001).

2.6. Statistics

All bioassays were performed in a completely randomized design and data was analysed for normality by Shapiro-Wilk test (α = 0.05) and for homoscedasticity by Barlett's test (α=0.05). After observing that data fitted these assumptions, data was analysed by ANOVA and the means were compared by Tukey test (P <0.05). Data was analysed and plotted using GraphPad Prism software v.9.0.

3. Results

In bioassays using artificial diet as substrate for feeding, only the treatment containing hemolymph serum proteins plus cells contents from parasitized larvae (Pc) resulted in negative effects over the development of larval *D. saccharalis*, given by a reduction of ~20% in larval viability ($F_{4,20}$= 3.0, P= 0.04) (Table 1).

Table 1. Immature development of *Diatraea saccharalis* fed with hemolymph serum and hemolymph serum plus cells contents of *D. saccharalis* larvae parasitized or non-parasitized by *Cotesia flavipes* in bioassays using artificial diet as feeding substrate. Control: anticoagulant buffer. Cs: hemolymph serum from non-parasitized larvae. Cc: hemolymph serum plus cell contents from non-parasitized larvae. Ps: hemolymph serum from parasitized larvae. Pc: hemolymph serum plus cell contents from parasitized larvae.

Treatments	Larval period (days)	Larval survival (%)	Pupal period (days)	Pupal survival (%)	Pupal weight (mg)
Control	32.4±0.9	93±3.0 a	10.2±0.1	85.8±2.1	184.9±2.5
Cs	31.9±0.3	88±5.6 a	10.3±0.1	94.3±2.7	178.7±6.3
Cc	33.0±0.5	83±4.1 a	10.5±0.1	94.5±2.7	182.6±5.9
Ps	31.2±0.3	83±3.0 a	10.3±0.1	96.4±2.3	177.3±3.5
Pc	33.0±0.6	75±3.2 b	10.2±0.1	96.1±1.6	196.1±9.6
ANOVA	$F_{4,20}$=1.7; P=0.2^{ns}	$F_{4,20}$=3.0; P=0.04	$F_{4,20}$=1.6; P=0.2^{ns}	$F_{4,20}$=2.5; P=0.1^{ns}	$F_{4,20}$=0.7; P=0.6^{ns}

Means ± standard errors. For each attribute, means followed by the same letter did not differ according to Tukey's test (P ≤ 0.05). ^{ns} = non significative according to ANOVA.

In bioassays using leaf discs as feeding substrates, a reduction of ~28.60% in larval survival, compared to control, was observed for the larvae feeding with leaves containing hemolymph serum plus cells contents from parasitized larvae (Pc) ($F_{4,20}$=5.1; P=0.005) (Fig. 1).

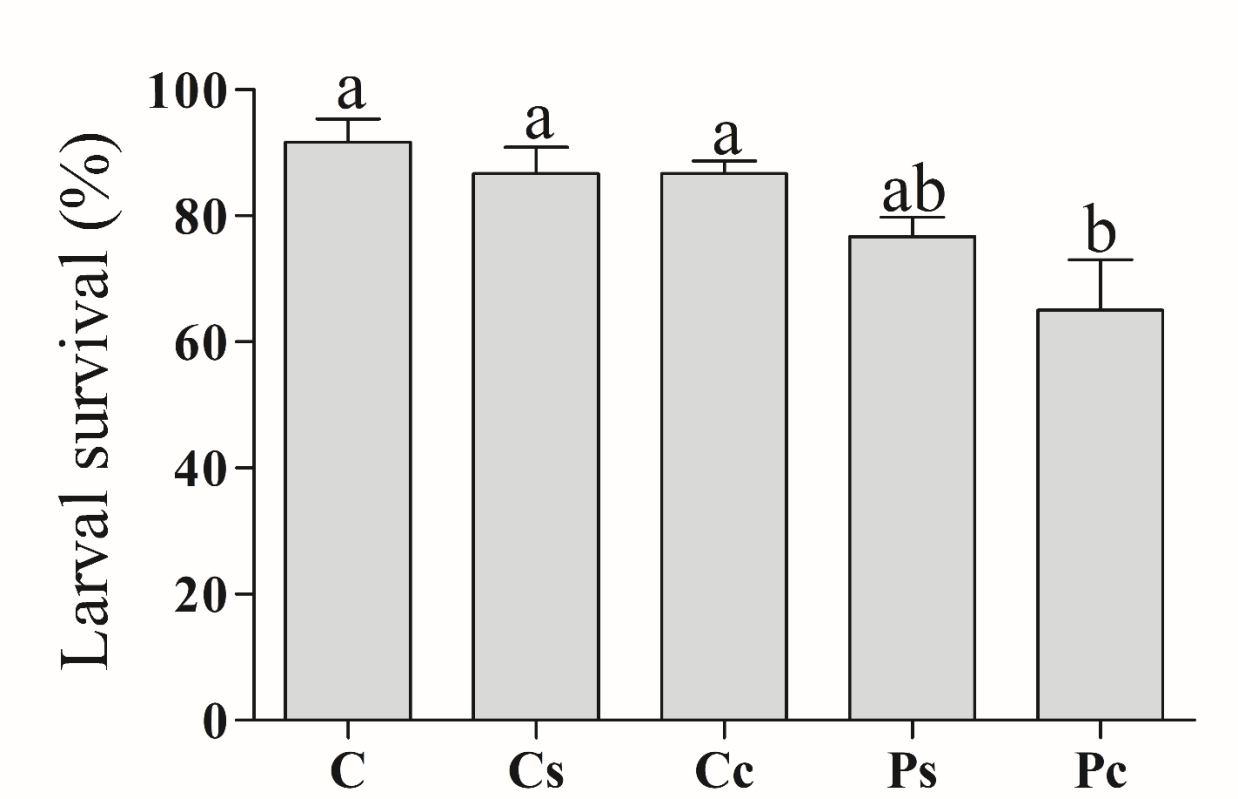

Figure 1. Survival (%) of *Diatraea saccharalis* neonates after 5 days feeding with hemolymph serum and hemolymph serum plus cells contents of *D. saccharalis* larvae parasitized or non-parasitized by *Cotesia flavipes* in bioassays using sugarcane leaf disks as feeding substrates**.** C: anticoagulant buffer. Cs: hemolymph serum from non-parasitized larvae. Cc: hemolymph serum plus cell contents from non-parasitized larvae. Ps: hemolymph serum from parasitized larvae. Pc: hemolymph serum plus cell contents from parasitized larvae. Bars represent ± standard error of the means. Means followed by different letters differ statistically by Tukey's test (P <0.05).

Given the negative effect of hemolymph serum plus cell contents from parasitized larvae (Pc) over the survival of *D. saccharalis* larvae, we fractionated this sample in

different molecular weight ranges using ultra-filters and applied the different fractions over both sugarcane leaf disc surfaces and offered to neonates.

Insect survival was not influenced by any of the fractions ($F_{6,35}$=1.1; P=0.37). However, the fractions containing molecules with molecular weight >100 kDa and among 3-10 kDa reduced ~33% leaf consumption by the neonates ($F_{6,35}$=4.7; P=0.001) (Fig. 2).

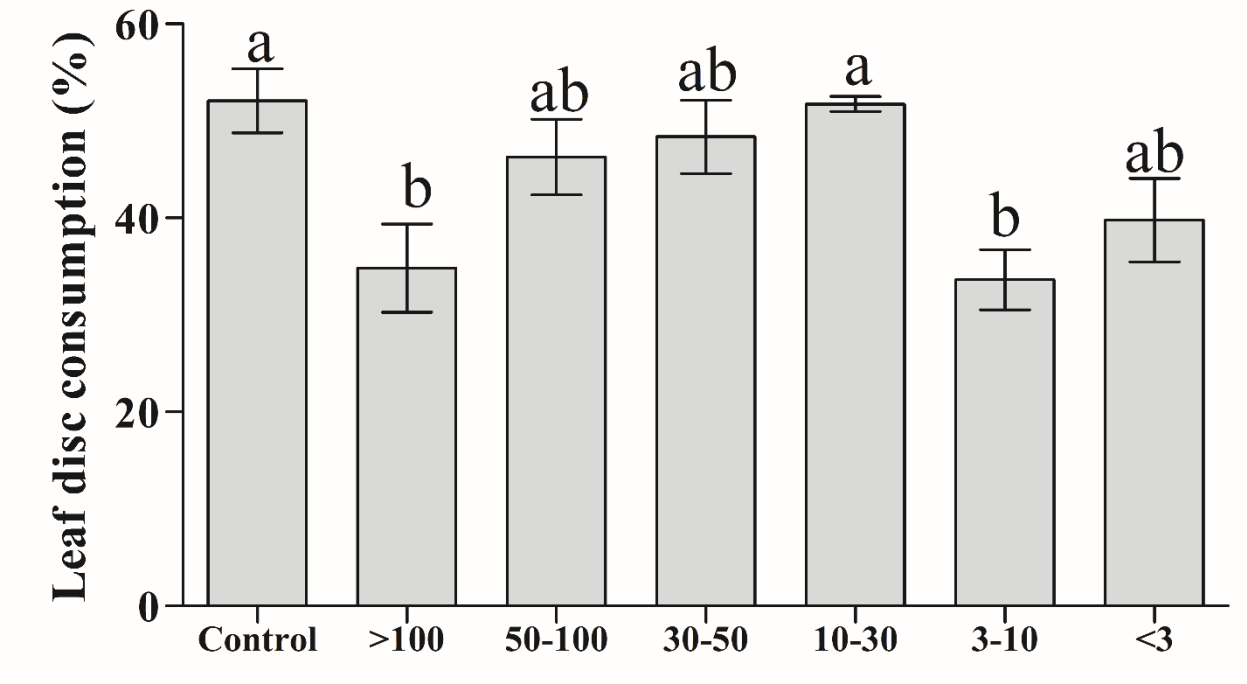

Figure 2. Leaf disk consumption (%) by *Diatraea saccharalis* neonates after 3 days feeding with hemolymph serum plus cells contents of *D. saccharalis* larvae parasitized by *Cotesia flavipes* fractionated by size. C: deionized water; <3: fraction containing molecules with less than 3 kDa; 3-10: fraction containing molecules within 3-10 kDa; 10-30: fraction containing molecules within 10-30 kDa; 30-50: fraction containing molecules within 30-50 kDa; 50-100 fraction containing molecules within 50-100 kDa; and >100: fraction containing molecules with molecular weight greater than 100 kDa. Bars represent ± standard error of the means. Means followed by different letters differ statistically by Tukey's test (P <0.05).

Protein profile of the fractions indicated differences between the fractions produced by fractioning hemolymph serum plus cell contents from parasitized and non-parasitized larvae. A decrease in proteins was observed in samples from parasitized larvae in the fraction <3 kDa (black boxes, Figure 3A). On the other hand, an increase in protein content was observed for the fraction 3-10 kDa prepared with hemolymph from parasitized larvae (red boxes Figure 3A). No clear differences among samples prepared

with hemolymph from parasitized or non-parasitized larvae were observed in the fractions 10-30, 30-50 and 50-100kDa (Fig. 3A and 3B). In the fraction >100 kDa, it was observed a parasitism-specific band with a molecular weight greater than 200 kDa (Figure 3B, black arrow).

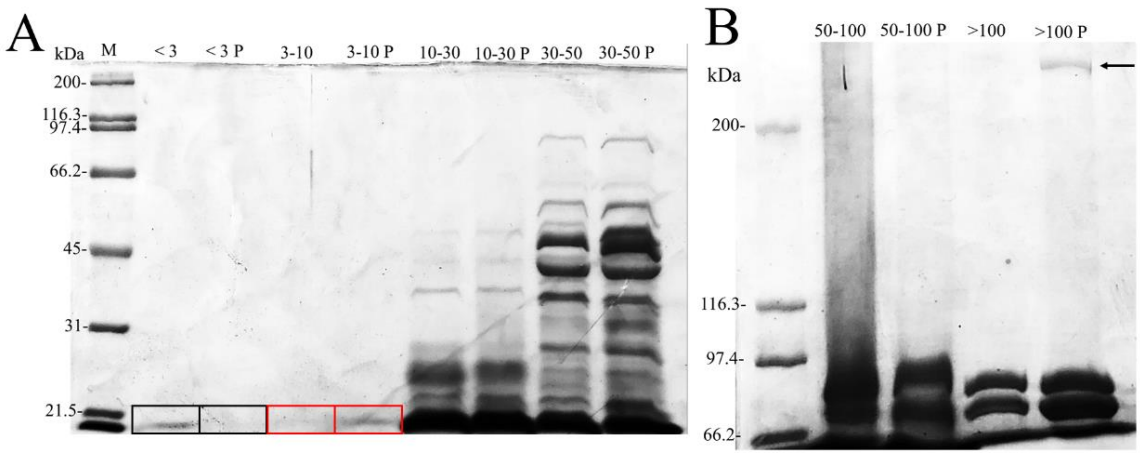

Figure 3. SDS-PAGE profile of molecular weight fractions from the hemolymph serum plus cell contents of *Diatraea saccharalis* larvae parasitized (Pc) or non-parasitized (Cc) by *Cotesia flavipes*. Proteins were stained with Coomassie Brilliant Blue R250. Molecular weight marker = SDS-PAGE Molecular Weight Standards, Broad Range (Bio-Rad, Hercules, CA, USA). **A**- protein profile of hemolymph submitted to electrophoresis in SDS-PAGE at 12% of polyacrylamide. **B**- protein profile of hemolymph submitted to electrophoresis in SDS-PAGE at 6% of polyacrylamide. <3: fraction containing molecules with less than 3 kDa; 3-10: fraction containing molecules within 3-10 kDa; 10-30: fraction containing molecules within 10-30 kDa; 30-50: fraction containing molecules within 30-50 kDa; 50-100 fraction containing molecules within 50-100kDa; and >100: fraction containing molecules with molecular weight greater than 100 kDa.

4. Discussion

It is known that endoparasitoids change the physiology of their hosts using a plenty of parasitism-related molecules that are released into the hemolymph of their hosts. These molecules derive from the venom, symbiotic viruses, and teratocytes of endoparasitoids (Yan et al., 2016; Chevignon et al., 2014; Gao et al., 2016) and, beyond host regulators, these factors may be exploited as insecticidal molecules.

The biotechnological exploitation of parasitoid-derived factors has been proven by different research teams. For example, parasitoid-derived molecules Cys-rich proteins

(Gill et al. 2006), chitolectins (Rossi et al. 2012; Merlin et al. 2020) and polydnavirus-derived proteins (Fath-Goodin et. al. 2006, Di Lelio et al. 2014, Kim et al. 2016, Wei et al. 2016a; Wei et al. 2016b) were tested and presented insecticidal activity against insect pests.

Our results indicated that the hemolymph serum of *D. saccharalis* parasitized by *C. flavipes* containing the cells contents presented insecticidal activity. Two factors lead us to include the contents of the cells in circulation in parasitized hosts: (*i*) hemocytes of hosts parasitized by Braconidae wasps are frequently infected by polydnaviruses and express host regulation proteins (Strand and Burke 2013) and (*ii*) at the age we collected hemolymph from parasitized *D. saccharalis* (6 days after parasitism), teratocytes, a specific parasitoid cell type involved in the production of host regulation molecules, and parasitoid larvae, which also produce host relegation peptides, are present in hemolymph (personal observation, Chapter III of this Thesis, Hartzer et al. 2005).

Protein fractioning profiles indicated changes in protein profile in parasitized and non-parasitized hosts. For instance, proteins with less than 3 kDa were not observed in parasitized hosts in the conditions that we used. Accordingly, this fraction from parasitized hosts did not result in larval mortality or leaf consumption. On the other hand, we observed a more intense protein band for the fraction 3-10 kDa in the hemolymph of parasitized hosts and a decreased rate of leaf consumption for larvae fed with leaves containing this protein fraction collected from parasitized hosts. The fractions 10-30, 30-50, and 50-100 kDa presented a similar SDS-PAGE profile and did not influenced larval survival or leaf consumption.

The fraction containing proteins >100 kDa presented two bands smaller than 100 kDa. These are known hemolymph storage proteins originally present in oligomeric form (> 100 kDa) formed by subunits (< 100 kDa) that dissociate into smaller units in SDS-PAGE (Koopmanschap et al. 1992, Grande et al. 2002). Moreover, fractioning proteins with > 100 kDa indicated the presence of a high molecular weight (> 200 kDa) specifically in the hemolymph plus cell contents of parasitized hosts.

The identification of parasitoid-derived molecules with insecticidal activity after ingestion is a promising tool for pest management. The mode of action of such molecules may be distinct from the mode of action of commercial pesticides or toxins expressed in

transgenic plants and may contribute not only in pest management, but also on Insect Resistance Management (Merlin et al. 2020).

Finally, parasitoid-derived molecules with host regulation activity can be exploited for the control of insect pests by, for instance, plant transgenesis with transgenes from symbiotic viruses and other teratocyte proteins (Maiti et al. 2003, Gill et al. 2006, Di Lelio et al. 2014, Kim et al. 2016, Wei et al. 2016a, Wei et al. 2016b, Merlin et al. 2020). The present work shed light on the potential of the parasitoid *C. flavipes* as a source of insecticidal molecules active against *D. saccharalis*. Studies over the identity and function of *C. flavipes* molecules related with host regulation are necessary before exploitation of this interesting biotechnological potential.

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Chapter III - An enemy within: Teratocytes released by the endoparasitoid *Cotesia flavipes* are master regulators of sugarcane borer host physiology

Abstract

Parasitoid wasps have evolved sophisticated mechanisms of host regulation that establish a favorable environment for the development of parasitoid immatures. While maternal venom and symbiotic virus-like particles are well-known mechanisms of host regulation, another less-studied mechanism concerns the action of secretory cells called teratocytes released by the growing embryo. Host regulation mediated by teratocytes still requires deeper characterization. We aimed to analyze the role of teratocytes released into hemolymph of the larval sugarcane borer *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae) by its biological control agent, the koinobiont endoparasitoid wasp *Cotesia flavipes* Cameron, 1891 (Hymenoptera: Braconidae). Teratocytes were released upon eclosion of parasitoid larvae at four days after parasitism (DAP) and increased in number and size until 6 DAP. Total *D. saccharalis* hemocyte viability was reduced immediately after parasitism until 2 DAP, while total hemocyte count was significantly lower from 3 DAP on, and phenol oxidase and lysozyme activity, two immune related enzymes, were disrupted compared to non-parasitized controls. Using mass spectrometry and sequencing of teratocyte RNA, we identified 61 polypeptides secreted by teratocytes and tracked their abundance over 0-10 DAP. Abundant teratocyte products included bracovirus-associated proteins, disulfide-rich peptides, and novel uncharacterized proteins. Most teratocyte products showed a significant increase in concentration leading up to parasitoid pupation. Our results provide insights into host regulation by teratocytes and reveal molecules that may be useful in biotechnology.

Keywords: Teratocyte, phenol oxidase, lysozyme, immune system, biological control

1. Introduction

The koinobiont endoparasitoid *Cotesia flavipes* Cameron, 1891 (Hymenoptera: Braconidae) is an important biological control agent of sugarcane borer, *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae). This parasitoid was introduced in the 1970s in Brazil and is currently used in approximately 3.3 million hectares, almost 40% of the sugarcane crops in the country (Parra 2014), representing an emblematic and successful biological program (Parra and Coelho, 2019).

To facilitate successful development of larvae, parasitoid wasps use several tools to alter the physiology and behavior of their hosts, conditioning the hemocoel environment according parasitoid needs. Non-parasitized hosts have well-developed immune systems that prevent invasion by microbes and parasites, consisting of cellular defenses mediated by hemocytes and humoral factors such as antimicrobial molecules and enzymes, that must be disabled by the parasitoid for successful survival inside the host. Accordingly, hemocyte function in *D. saccharalis* is strongly altered after parasitism by *C. flavipes* (Kryukova et al. 2015, Burke and Strand 2014, Hillyer 2016). The activity of enzymes such as lysozymes which are antimicrobial cell-wall-degrading enzymes, and phenol oxidases (PO), which melanizes foreign bodies, may also be affected (Nakhleh et al. 2017, Callewaert and Michiels 2010, van Herreweghe and Michiels 2012).

These changes induced in host physiology, termed host regulation, depends on molecular factors originating in maternal, embryonic or post-embryonic parasitoid tissues (Strand 2014, Ali et al. 2015). One set of maternal factors for host regulation is the venom injected by the parasitoid female into the host hemolymph during oviposition (Asgari and Rivers 2011, Moreau and Asgari 2015). Along with the venom, some parasitoids may inject symbiotic viruses that have strong effects in host regulation (Gundersen-Rindal et al. 2013).

An additional source of regulation factors are specialized cells, teratocytes, that are released from the embryonic serosa into the hemolymph of the host during larval eclosion (Dahlman 1990, Strand 2014). After release, teratocytes develop and disperse throughout the hemocoel of the parasitized host (Dahlman 1991, Burke and Strand 2014) increase substantially in size, but do not perform significant cell division and exhibit few external morphological changes according parasitoid development (Hotta et al. 2001).

While parasitoid wasps use different evolutionary strategies to impair the host immune system (Burke and Strand 2014), in many species, teratocytes may be the main source of host regulatory proteins after parasitoid larval hatching. Teratocytes induce various physiological changes in the host, including immunosuppression (Rana et al. 2002, Ali et al. 2015), control of endocrine system (Ignesti et al. 2018; Pennacchio et al. 1994), changes in metabolic pathways (Cônsoli et al. 2001, Merlin and Cônsoli 2019), control of protein synthesis (Kadono-Okuda et al. 1998), anomalies in metamorphosis (Shi et al. 2015), reprogramming of neuropeptides (Shi et al. 2016), and enzymatic degradation and disfunction of host fat bodies (Nakamatsu et al. 2002, Salvia et al. 2019). Disabling of the host immune system renders vulnerability against microbial or other parasitic invasions that might interfere in successful parasitoid development. Thus, another important role of the teratocyte is the development of a new “immune system”, by producing and releasing antimicrobial peptides in host hemocoel (Gao et al., 2016).

Despite the potential of teratocytes as a source of molecules for application in biotechnology, and the availability of several studies investigating the biology of host-parasitoid interactions using transcriptomics and other techniques (Gao et al. 2016, Burke and Strand 2014, Ali et al 2015), few details are known about the identity and the temporal release of molecules produced by teratocytes. Furthermore, a transcriptome coupled to proteomic analyses has never performed on teratocytes to clarify the proteinaceous produced by this cell type that are released into the host hemolymph. Here, we report a temporal study from day 0 until day 10 of the interaction of *D. saccharalis* parasitized by *C. flavipes*. First, we analyzed immune factors of the host by measuring hemocyte number and viability, and PO and lysozyme activities. We also observed the dynamics of teratocytes by measuring date of release, number and size of this cell type during parasitism. Then, we performed a detailed proteomic analysis of the hemolymph of *D. saccharalis* larvae parasitized by *C. flavipes* and connected the results of proteomics with the transcriptomic of *C. flavipes* teratocytes. This study sheds light on the complex interactions between parasitoid and host over the time and correlating the identity of protein factors throughout parasitism highlighting the role of teratocytes in during parasitism.

2. Material and Methods

2.1. Insect rearing

Immatures of *D. saccharalis* and cocoons of *C. flavipes* were kindly donated by a local biofactory (21º21'23"S, 48º3'48"W), São Paulo-Brazil. Sugarcane borer larvae were kept in Petri dishes (90 mm diameter; 15 mm height) containing portions of artificial diet (Parra 2001). Parasitoid cocoons were kept in plastic containers with a lid, where adult emergence and mating also took place. Insects were kept under controlled conditions (25±1°C, 70±10% relative humidity, and 12 h photophase).

2.2. Parasitism

Fifth instar *D. saccharalis* were individually parasitized by a single parasitoid wasp. Parasitoids were removed after parasitism to avoid a second oviposition by the same wasp. As control treatment, larvae of the same instar were kept separated without parasitism.

2.3. Hemolymph collection

Hemolymph of parasitized and non-parasitized insects were daily collected until, respectively, the tenth (parasitoid larval emergence from the hosts) and seventh or eighth day after parasitism (DAP). This difference (7^{th}-8^{th} days) is because most non-parasitized insects pupated after the seventh or eighth DAP, whereas pupation is disabled in parasitized insects. For hemolymph collection, the last larval abdominal legs were severed with ophthalmic scissors, and the extravasated hemolymph was collected.

2.4. Circulating cells analysis

Prior to cell counting, hemolymph was 10-fold diluted in anticoagulant buffer (0.098 M NaOH, 0.15 M NaCl, 0.017 M EDTA, 0.046 M citric acid) (Hotta et al. 2001). Total hemocyte count (THC) present in *D. saccharalis* hemolymph was estimated using a Neubauer chamber (HBG® 9020-01) and a phase-contrast microscope (Zeiss® Axio Imager A2; 40x magnification). Cell counting was performed according technical recommendations (Bastidas 2009). The same procedure was used for the total

teratocytes count (TTC). The diameter of the teratocytes was measured using the phase-contrast microscope (Zeiss® Axio Imager A2; 40x magnification) coupled to a micro ruler.

Hemocyte viability was evaluated using Trypan Blue reagent (Sigma-Aldrich®), which penetrates dead cells, giving a distinguishable dark blue colour to them. For hemocyte viability, extravasated hemolymph (10 µL) was immediately diluted 5-fold in deionized water at room temperature and mixed with the same volume of Trypan Blue reagent. The solution was placed in a microscope slide, covered with a cover glass and observed under a phase-contrast microscope (Zeiss® Axio Imager A2; 40x magnification). The following formulas were used for cell viability estimation:

$$THC\ \mu L^{-1} = [(\sum cells \times dilution \times 10{,}000)/\sum quadrants]/1000$$

$$DHC\ \mu L^{-1} = [(\sum dark\ cells \times dilution \times 10{,}000)/\sum quadrants]/1000$$

$$HV = [(DHC\ \mu L^{-1} x100) / THC]-100$$

Where:

$THC\ \mu L^{-1}$ = Total hemocytes count μL^{-1}

$DHC\ \mu L^{-1}$ = Dead hemocytes count μL^{-1}

HV= Hemocytes Viability (%)

For the experiments of THC and TTC we used five replicates, each composed by three independent insects. For cell viability, we considered the average counting of viable/dead cells from four quadrants of Neubauer Chamber for each replicate. Parasitized and non-parasitized *D. saccharalis* hemolymph was collected daily from 0 until 10 DAP. Results obtained from parasitized and non-parasitized larvae were compared by unpaired *t*-test ($P<0.05$) using Prism 5 (GraphPad Software Inc., La Jolla, CA).

2.5. Hemolymph protein samples

For the analysis of total proteins, samples were firstly centrifuged to remove the cell fraction (800 *g*; 5 min; room temperature). The supernatants (hemolymph serum) were transferred to a new Eppendorf tubes, the pellets were discarded, and hemolymph plasmas were diluted 100-fold in deionized water. Total hemolymph plasma proteins of *D. saccharalis* were measured using Bradford Reagent (Sigma-Aldrich®) and bovine

serum albumin as standard (Bradford 1976). Five replicates composed by three independent insects each were used for the total protein quantification in the hemolymph of parasitized or non-parasitized *D. saccharalis* larvae sampled at 24 h intervals from 0 to 10 DAP. Protein contents from parasitized and non-parasitized insects were compared with unpaired *t*-test ($P < 0.05$) using Prism 5 (GraphPad Software Inc., La Jolla, CA).

2.6. Phenol oxidase and lysozyme activities

Phenol oxidase (PO) activity was measured by adding 10 µL of hemolymph serum pooled from three insects to 990 µL of substrate (5 mM L-DOPA, 10 mM sodium cacodylate, 5 mM $CaCl_2$ pH 7.0) in a cuvette. After a quick homogenization by pipetting, the cuvette was placed into a spectrophotometer (Metash®) and PO activity was measured by continuously reading the absorbance at 490 nm in intervals of 2 min during 20 min. PO activity was calculated using the linear phase of the readings (Absorbance *vs* time; $R^2 \geq 0.99$). One unit of PO (U) was considered as the amount of enzyme necessary to increase 0.001 units of absorbance at 490 nm per min.

Lysozyme assay was performed according the method described by Chhabra et al. (2007), with adaptations. Briefly, lyophilized cells of *Micrococcus lysodeikticus* (0.015% w/v) (Sigma Chemicals St. Louis) were initially suspended in potassium phosphate buffer (0.05 M; pH 6.4). Subsequently, 485 µL of the *M. lysodeikticus* suspension were added to 15 µL of *D. saccharalis* hemolymph serum in a quartz cuvette. After a quick homogenization by pipetting, the cuvette was placed into the spectrophotometer and absorbance was continuously measured at 570 nm in 2 min intervals during 30 min. One unit of lysozyme (U) was defined as the amount of enzyme necessary to increase 0.0001 units of absorbance at 570 nm per min.

Eight to ten replicates composed by pooling the hemolymph serum from three parasitized or non-parasitized *D. saccharalis* larvae each were used for the experiments of PO and lysozyme activities. Samples were collected from 0 to 10 DAP at 24 h intervals. The activities of parasitized and non-parasitized insects at the same collection days were compared with unpaired *t*-test ($P < 0.05$) using software Prism 5 (GraphPad Software Inc., La Jolla, CA).

2.7. Sample collection for transcriptome and teratocytes filtering

Hemolymph of parasitized and non-parasitized insects were daily collected until the 8 and 10 DAP, respectively. As control, we used unparasitized last instar larvae. For hemolymph collection, one of last abdominal leg of externally sterilized *D. saccharalis* larvae was severed with ophthalmic micro scissors and the extravasated hemolymph was immediately collected and 20 mM of phenylthiourea was added to avoid melanization (Ryazanova et al. 2012). Subsequently, for removing cells and debris, hemolymph samples were centrifuged (1000 *g*, 5 min, 4°C) and stored at -20°C for proteomic analysis.

Most *C. flavipes* 3 days old teratocytes (6 DAP) were bigger than 30 μm, several times bigger than host hemocytes, which facilitates their isolation by filtration. Thus, for teratocyte collection, we firstly externally sterilized *D. saccharalis* larvae with 70% ethanol and, with a sterile ophthalmic micro scissors, we severed one of the larval abdominal leg and made a gentle pressure over larvae to induce hemolymph extravasation. Immediately, hemolymph was pipetted into a 1.5 mL tube and centrifuged (5 min, 400 *g*, 4°C). Supernatant was discarded and the pellet containing cells were resuspended in phosphate buffer saline (PBS, 100 mM, pH 7.0). For teratocyte collection, we used as a filter a voile square with ~30 μm pores. Teratocytes were filtered three times, with PBS as washing buffer, and teratocyte purity was verified by phase contrast microscopy (Zeiss® Axio Imager A2; 40x magnification). Due to the higher number of cells per unit of hemolymph, we collected 3, 4 and 5 day old teratocytes (6, 7, 8 DAP) from 100 *D. saccharalis* larvae each day.

2.8. Teratocytes transcriptomics

Immediately after collected, teratocytes suspensions were centrifuged (5 min, 400 *g*, 4°C) and the supernatant discarded. Teratocyte total RNA was extracted with TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). RNA concentration and quality were verified using the ration of absorbances at 260/280 nm and 260/230 nm on a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Construction of teratocyte RNA-Seq libraries was performed by Sequencing Facility at the Institute for Molecular Bioscience at The University of Queensland,

Brisbane, Australia. A dual-indexed library was constructed with the TruSeq-3 Stranded mRNA Sample Prep Kit (Illumina) with oligo (dT) selection and an average insert size of 180 base pairs. 150-cycle paired-end sequencing was performed on an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA), yielding 23,834,838 paired-end reads. Adapter trimming of demultiplexed raw reads was performed using fqtrim (Pertea G. Fqtrim: v0. 9.4 release. 2015. 10.5281/zenodo.20552), followed by quality trimming and filtering using prinseq-lite (Schmieder and Edwards, 2011). Error correction was performed using BBnorm tadpole, which is a part of the BBtools package. Trimmed and error-corrected reads were assembled using Trinity (version 2.4.0) (Haas et al., 2013; Grabherr et al., 2011) with a k-mer length of 31 and a minimum k-mer coverage of 2. Assembled transcripts were annotated using a BLASTx (Altschul et al. 1990) search (E value setting of $1e^{-3}$) against the Swiss-Prot database. Estimates of transcript abundance was performed using the RSEM (Li and Dewey 2011) plugin of Trinity (align_and_estimate_abundance). Using TransDecoder, transcripts were translated and filtered to open-reading frames (> 30 amino acids), and the resulting FASTA file was used, together with a list of common protein contaminants, for protein identifications based on mass spectral data.

2.9. Protein samples preparation and mass spectrometry

Cell-free hemolymph collected from 0 to 10 DAP was used for proteomic analysis. We performed LC-MS/MS of reduced, alkylated and trypsinized samples, using ~100 μg of protein incubated for 1 h at 37°C in a reducing/alkylating buffer (1% 2-iodoethanol, 0.25% triethylphosphine, 48.75% acetonitrile, 50 mM ammonium bicarbonate pH 11.0). For digesting reduced/alkylated samples, they were dried by vacuum centrifugation and reconstituted in a digestion buffer (40 mM ammonium bicarbonate pH 8.0, 10% acetonitrile) with 20 ng/μL trypsin for 1 h at 37°C (Sigma Aldrich, St. Louis, CA, USA). Subsequently, samples were dried in a vacuum centrifuge and reconstituted in 1% formic acid. The digestion step was omitted for reduced/alkylated samples, and both reduction/alkylation and digestion steps were omitted for the untreated samples.

For liquid chromatography (LC), hemolymph samples were loaded into a Zorbax 300SB-C_{18} column (Agilent, Santa Clara, CA, USA) with outflow coupled to a SCIEX 5600

Triple TOF (Framingham, MA, USA) mass spectrometer (MS) equipped with a Turbo V ion source. Elution time was set 70 min into a gradient of 1-40% solvent B (90% acetonitrile and 0.1% formic acid) in solvent A (0.1% formic acid) at a flow rate of 0.2 mL/min. MS^1 scans were collected between 350–2200 *m/z*, and the MS2 scans between 350–1500 *m/z*. Peptides with +2 to +5 charge and >100 counts/s were selected for MS^2 fragmentation, excluding adjacent peaks within 2 Da. Scans were obtained with an accumulation time of 250 ms and a cycle of 4 s. The generated mass spectra were searched against the transcriptome library obtained from *C. flavipes* teratocytes using the Paragon 4.0.0.0 algorithm in Protein Pilot 4.0.8085 software (SCIEX, Framingham, MA, USA). To identify teratocyte-expressed proteins in samples of hemolymph from parasitized insects, we removed all proteins that could be detected in hemolymph of non-parasitized insects. Each protein identification was reviewed manually to obtain the correct ORF by re-mapping trimmed reads in Geneious software, and low-quality protein identifications were removed. The threshold used was one or more peptides with >95% confidence (*p*>95%) with signal peptide with D-score >0.7 according results obtained from SignalP 4.1 (Nielsen 2017; Petersen et al. 2011). Data were compared between untreated, reduced/alkylated, and reduced/alkylated/trypsinized datasets to check for the presence of post-translational modifications (PTMs) and confirm the *N*- and *C*-termini of each detected polypeptide. These data, together with the results of the SignalP 4.1 algorithm were used to determine/predict the mature form for each detected polypeptide.

2.10. Protein abundance estimates

The exponentially modified protein abundance index (emPAI), is an estimate of protein abundance obtained from LC-MS/MS experiments (Ishihama et al. 2005). The emPAI65 is an adjustment in the formula that makes the index more accurate (Kudlicki 2012). For calculating emPAI65 of our data set, we constructed a file containing the putative mature forms of all teratocyte-derived polypeptides (*i.e.*, without signal peptides) which was re-searched against LC-MS/MS datasets. To obtain the expression pattern of proteins during parasitism, we performed emPAI65 for all DAPs using the formula $emPAI65=6.5^{\log(cov95\%/100)-1}$.

2.11. Alignment and protein modelling

For structural analysis of teratocytes transcripts, we searched for homology by BLASTp in NCBI protein database (http://blast.ncbi.nlm.nih.gov/, June 2020). The *C. flavipes* teratocyte Host Translation Inhibitory Factor (*Cf*tHTIF), the highest teratocyte-expressed protein detected in the hemolymph of parasitized larvae, was used to identify additional HTIFs homologs. We closest HTIF homologs were found in bracoviruses simbionts of *Cotesia congregata* (Hymenoptera: Braconidae), (YP_009665795.1, YP_009665794.1, and YP_009665793.1) and *Cotesia plutellae* (Hymenoptera: Braconidae) ABI16035.1. For *C. flavipes* teratocyte Defensin (*Cf*tDef), the highest expressed antimicrobial factor observed in the hemolymph of parasitized larvae, we selected *Cotesia vestalis* (Hymenoptera: Braconidae) AGE89782.1, *Bemisia tabaci* (Hemiptera: Aleyrodidae) XP_018901504.1, *Galleria melonella* (Lepidoptera: Pyralidae) XP_026765201.1, *Leptidea sinapis* (Lepidoptera: Pieridae) VVC93999.1, *Helicoverpa armigera* (Lepidoptera: Noctuidae) ADR51145.1, *Manduca sexta* (Lepidoptera: Sphingidae) ADQ00386.1 and *Chloridea virescens* (Lepidoptera: Noctuidae) ACR78445.1 for structural analysis. Multiple alignment was performed using MAFFT Version 7 (Katoh et al. 2019) and edited by Bioedit (www.mbio.ncsu.edu). In addition, to characterize a highly expressed defensin from teratocyte, as previously described (Wang et al. 2014, Gao et al. 2016), we virtually modeled our sequence against Protein Data Base (https://www.rcsb.org/). For molecular modeling we used the automated SWISS-MODEL workspace (http://swissmodel.expasy) and for editing the model, Discovery Studio 2020 (Biovia).

3. Results

3.1. Parasitism alters immunity features of host hemolymph

To investigate the effects of parasitism on host hemocytes, we quantified hemocyte number and viability from 0 until 10 DAP (Figs. 1A and 1B). Hemocyte viability was reduced in parasitized larvae at 0 (P=0.0003), 1 (P=0.0201) and 2 (P=0.0205) DAPs. Only at 5 DAP, hemocyte viability was higher (P=0.0053) in parasitized larvae than in non-parasitized larvae (Fig 1A). From 3 DAP on, total hemocyte counting (THC) was lower (P=0.0014, 0.0192, 0.0024, 0.0255, 0.0122 at 3, 4, 5, 6 and 7 DAPs, respectively)

in parasitized than non-parasitized larvae (Fig. 1A). Total protein contents was also impaired in parasitized larvae, as indicated by a significant reduction of the total protein concentration in the hemolymph serum of *D. saccharalis* parasitized by *C. flavipes* from 2 DAP onwards compared with non-parasitized larvae (P= 0.016, <0.001, 0.011, 0.009, <0.001, 0.002, <0.001 and 0.011 for DAPs 2, 3, 4, 5, 6, 7 and 8 respectively; Fig. 1B).

Humoral immune responses were affected by parasitism, with a significant reduction in PO activity in parasitized larvae at the 2 (P=0.03) and 3 (P<0.001) DAP (Fig. 1C). At 6 DAP, PO activity of parasitized larvae was significantly higher than in non-parasitized (P<0.001), a status that changed again at 7 (P=0.009) and 8 (P<0.001) DAP (Fig. 1C). Until 4 DAP, no changes in lysozyme activity was detected among parasitized and non-parasitized larvae. At 5 DAP, we observed a significant reduction in lysozyme activity of parasitized larvae (P=0.001). At 7 (P<0.001*)* and 8 (P=0.005) DAP, lysozyme activity in the hemolymph of parasitized host was also significantly lower than in non-parasitized larvae. At 6 DAP, lysozyme activity in the hemolymph of parasitized larvae was significantly lower than that observed in the hemolymph of non-parasitized hosts (P=0.042) (Fig. 1D).

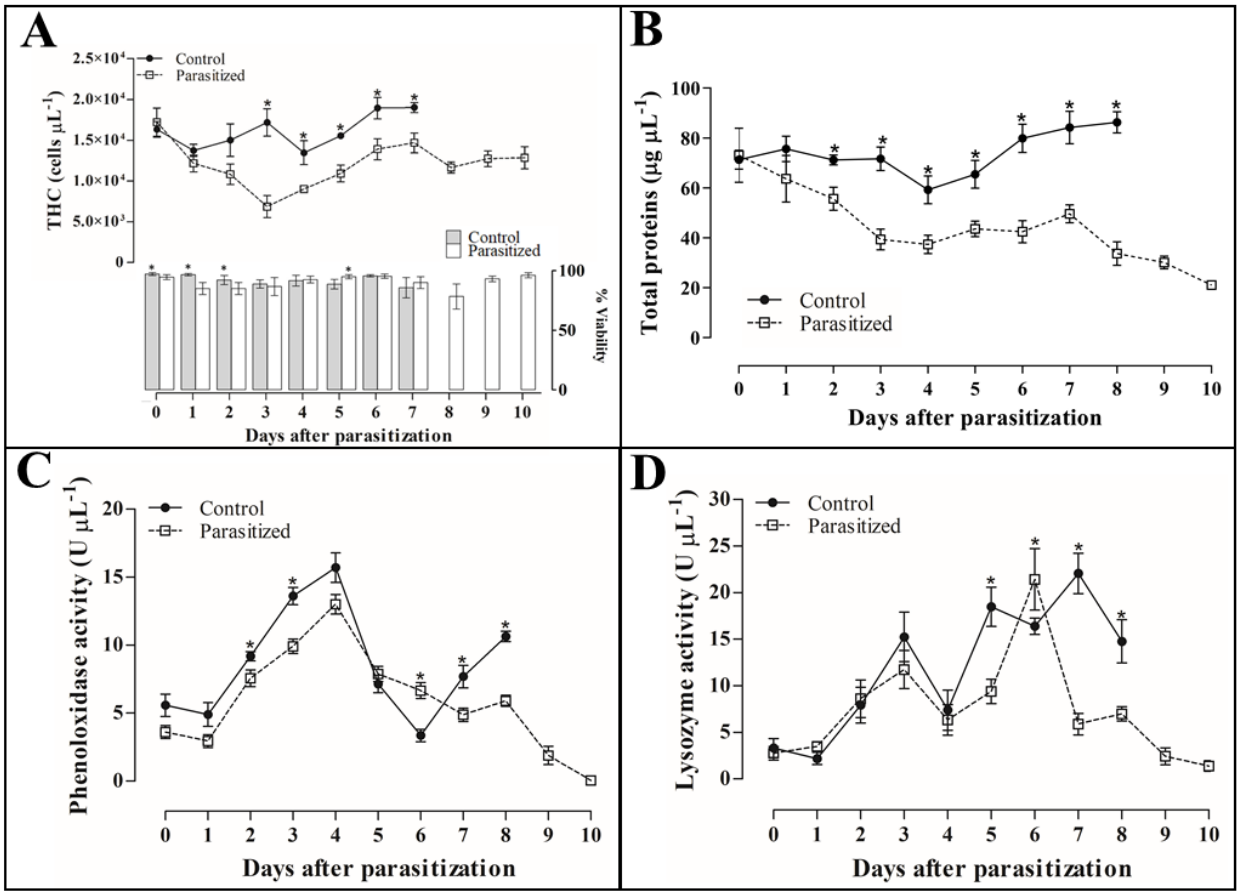

Figure 1. Time-course changes (0-10 days after parasitism) in the hemolymph contents of *Diatraea saccharalis* parasitized by *Cotesia flavipes*. (A) Total hemocytes count (hemocytes µL^{-1}) and hemocyte viability (%) in the hemolymph of *D. saccharalis* parasitized by *C. flavipes*. (B) Protein contents (µg of protein µL^{-1}) in the hemolymph of *D. saccharalis* parasitized by *C. flavipes.* (C) Phenol oxidase activity (Units µL^{-1}) in the hemolymph of *D. saccharalis* parasitized by *C. flavipes*. (D) Lysozyme activity (Units µL^{-1}) in the hemolymph of *D. saccharalis* parasitized by *C. flavipes*. *Indicates statistical difference among parasitized or non-parasitized larvae at the same day (t-test; *P*<0,05). Data are presented as means ± SEM.

Teratocytes from *C. flavipes* started to be detected in the hemolymph of *D. saccharalis* at 4 DAP, with a maximum number of approximately 136 cells µL^{-1} at 6 DAP. After 6 DAP on, a decrease in teratocyte number was observed, showing approximately 50 cells µL^{-1} at 10 DAP (Fig. 2). The diameter of *C. flavipes* teratocytes increased gradually after released in the hemolymph of the host, ranging from approximately 32.5 µm just after being released at 4 DAP until approximately 95 µm from 6 until 10 DAP (Fig. 2).

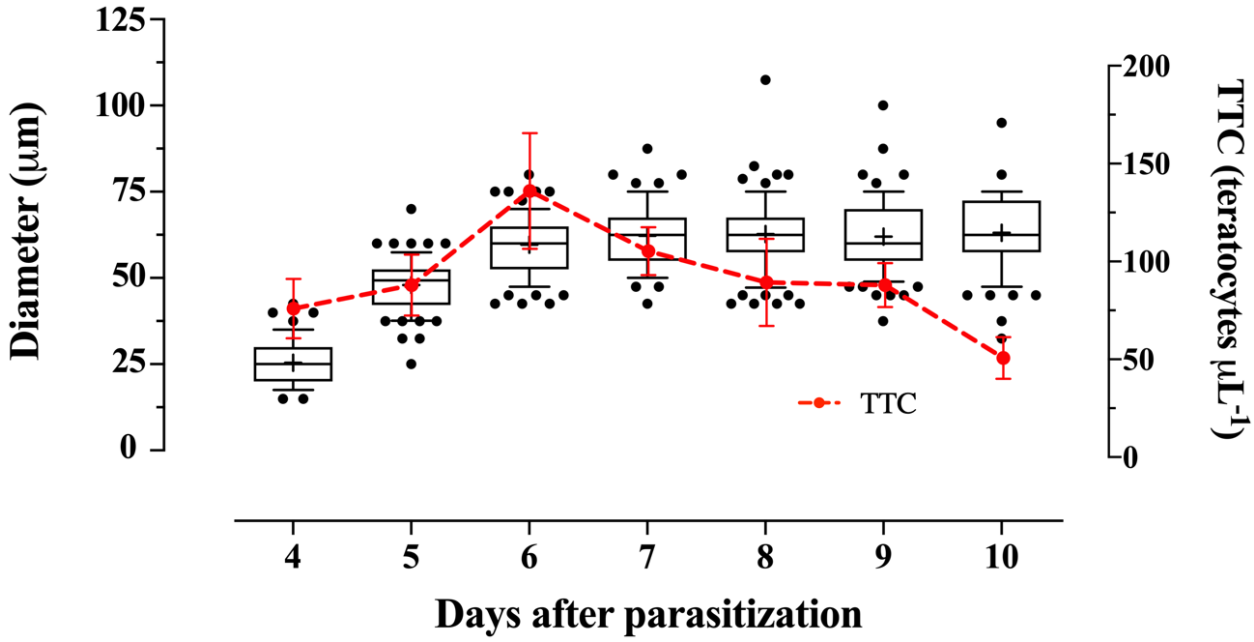

Figure 2. Size and number of the teratocytes of *Cotesia flavipes*. Diameter of teratocytes (μm) released by *C. flavipes* parasitizing *Diatraea saccharalis* is represented by boxplot (left y axis). Horizontal bar in each box represents the median. The upper and bottom portions of each box show the 25th and 75th percentile points, respectively. Vertical bar extending from each box are for the 90th percentile. Each point outside the box represents outliers of less than 10 or more than 90 percentile points. Total teratocytes count (TTC μL^{-1}) of *C. flavipes* in the hemolymph of *D. saccharalis* during parasitism is represented by red line (right y axis). Data are presented as cells μL^{-1} of hemolymph (means ± SEM).

3.2. Proteotranscriptomics reveals primary structure and temporal accumulation of teratocyte products during parasitism

To identify the transcripts from the teratocytes of *C. flavipes*, we generated a transcriptome using teratocytes from *C. flavipes* collected at 6, 7 and 8 DAP (3-5 days after teratocyte release). Following, we analyzed the proteome of hemolymph samples from *D. saccharalis* parasitized by *C. flavipes* from 0 until 10 DAP. The proteome analyses presented several technical challenges, such as the ubiquitous presence of proteins from non-parasitized hosts on both treatments (parasitized and non-parasitized). To remove the proteins from non-parasitized hosts, we subtracted all protein dataset detected in non-parasitized hosts from the protein dataset detected in the hemolymph samples from parasitized hosts. Additionally, we only selected the spectral data from proteins of the

hemolymph of parasitized *D. saccharalis* larvae that corresponded to their respective coding sequences obtained in the teratocyte transcriptome.

This process yielded a total of 57 polypeptides observed only in the hemolymph of parasitized *D. saccharalis*, *i.e.* parasitism-specific polypeptides (Fig.3). The abundances of transcripts encoding detected polypeptides within teratocyte-derived RNA ranged from 2 until 54,750 transcripts per million (TPM), with an average of 200 TPM. Annotation and amount of each amino acid sequence detected is summarized in Fig. 4. Many of the annotated proteins were classified as viral-derived or as peptides (including antimicrobial), enzymes, antimicrobial proteins, serine protease inhibitors (serpins), and others (Fig. 3).

Peptides (15.79% of detected sequences accounting for 19.91% of normalized transcripts) and virus-associated proteins (17.54 of detected sequences accounting for 28.76% of normalized transcripts) were the major secreted products of teratocytes. For 40.35% of the obtained sequences, no significant homology was detected. Antimicrobial components and serpins were detected mainly in later DAP (12.28% and 3.51% of detected sequences accounting for 4.00% and 3.04% normalized transcripts, respectively).

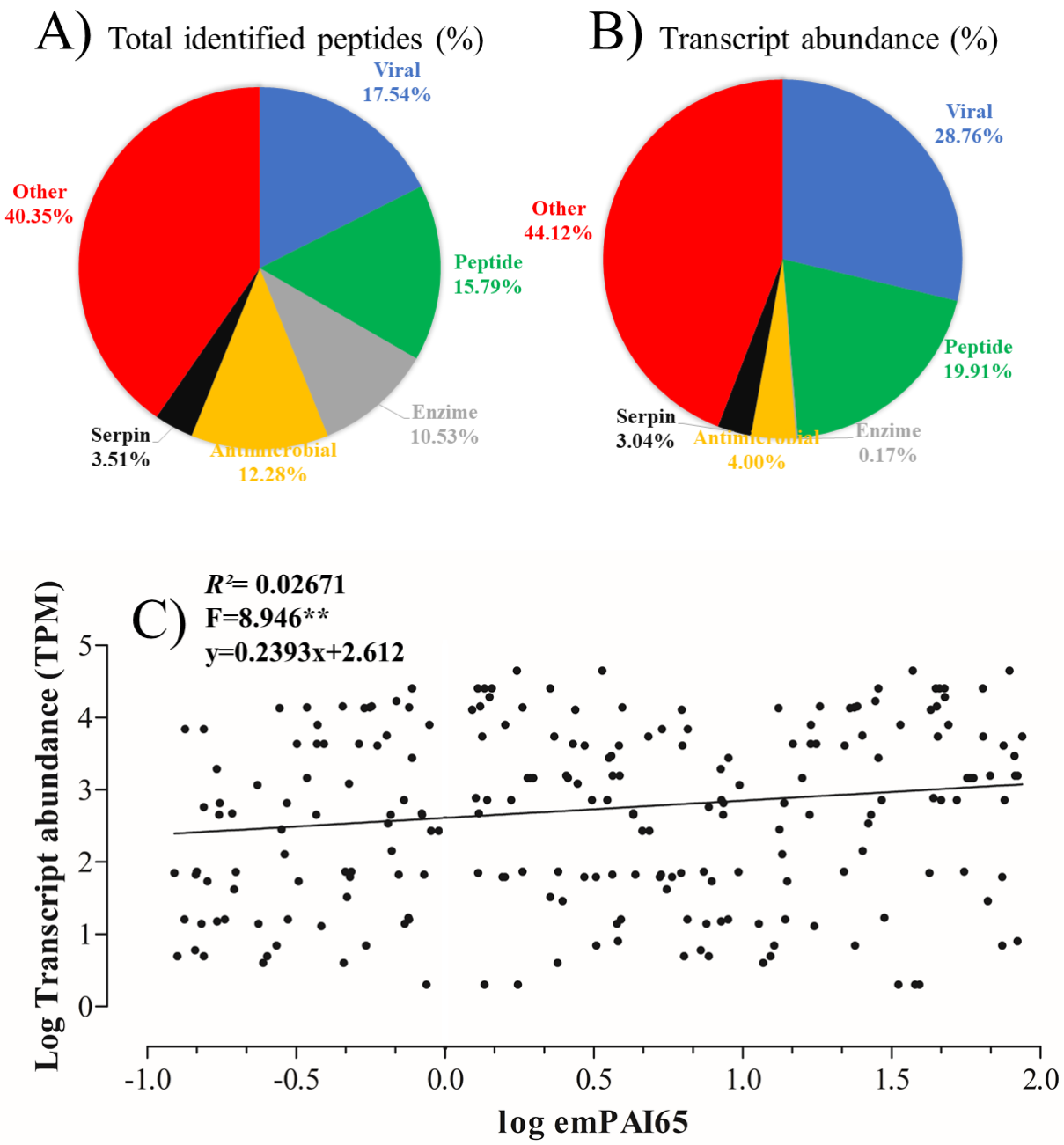

Figure 3. Abundance of proteins and peptides secreted by the teratocytes of *Cotesia flavipes*. (B) Percentage of total amount of teratocytes proteins detected by our protetranscriptomic approach. (C) Percentage of the abundance of the proteins. Data are presented in number of proteins with complete sequence detected and normalized transcript abundance according the categories viral, peptide, enzyme, antimicrobial, serpin and other. A) EmPAI of *C. flavipes* teratocyte-related and viral proteins present in the hemolymph of *Diatraea saccharalis*.

To better understand the time-course secretion of these products, we generated estimatives of protein abundance using emPAI values calculated from our LC-MS/MS datasets, for each sample from 0-10 DAP. Although the Pearson correlation coefficient of emPAI *vs* mRNA was considered moderately positive (Pearson's coefficient= 0.1634; R^2= 0.0267; 95%CI= 0.08249-0.3961; *P*=0.0030**), this is not surprising because our RNA sample contained teratocytes collected at 5-7 DAP whereas our emPAI values are pooled across many days (0-10 DAP) wherein protein abundances in hemolymph change. Better correlations are obtained correlating emPAI values from 0-10 DAP with transcriptome TPM values, suggesting that transcription during 5-7 DAP drives protein abundance throughout the parasitism period.

The results of proteomics of the hemolymph of parasitized *D. saccharalis* larvae coupled to the transcriptomics of teratocytes collected at 5-7 DAP indicate the expression of teratocyte-specific proteins mostly produced just after teratocyte release at 4 DAP until 10 DAP. Antimicrobial peptides were produced in almost all days after teratocyte release. Hydrolases were produced mainly at 10 DAP. One serpin (serine proteases inhibitor) was detected at 4 until 10 DAP and the other serpin was detected only at 10 DAP. Viral proteins associated with the symbiotic polydnavirus of *C. flavipes* were detected at 4 DAP along with teratocyte release from the eggs but viral proteins were also detected at 1-3 DAP, a period in parasitism before teratocyte release. From teratocyte release (4 DAP) until the end of the parasitic phase (10 DAP) it was detected one knottin, one ion channel inhibitory toxin, one von Willebrand factor, and one peritrophin. One Lectin (C-type domain) was detected at 4 DAP. Specifically at 10 DAP (end of parasitic phase), it was detected another von Willebrand factor, one apolipoprotein, one cystatin domain, one cystatin-like protein, one involucrin, one astakine-like protein, one lipocalin, one cystatin, and one stigma-specific protein (Fig. 4).

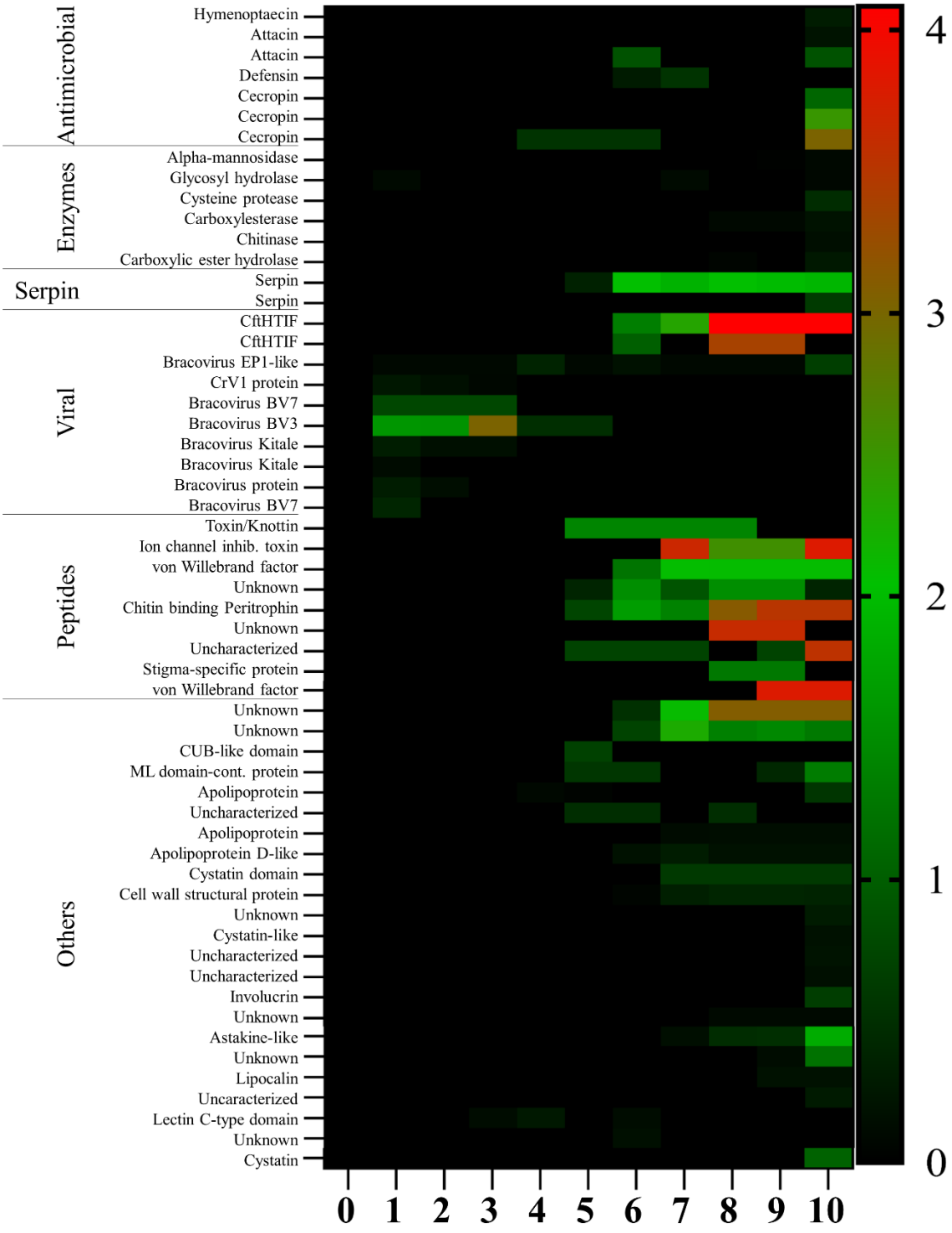

Figure 4. Heatmap showing exponentially modified protein abundance index (emPAI65) for teratocyte detected proteins into *Diatraea saccharalis* hemolymph for each day after parasitism (DAP). To the left, the putative function of identified proteins. The bar to the right represents the emPAI65 gradient of the peptides. The X axis means days after parasitism (DAP) from 0-10. Teratocytes started to be released at 4 DAP.

The transcript with the highest abundance (# TPM) in our teratocyte transcriptome (Supl. Table 1; Fig. 4) encodes a Host Translation Inhibitory Factor observed in the genome of symbiotic polydnabracoviruses (CftHTIF) (Fig. 5).

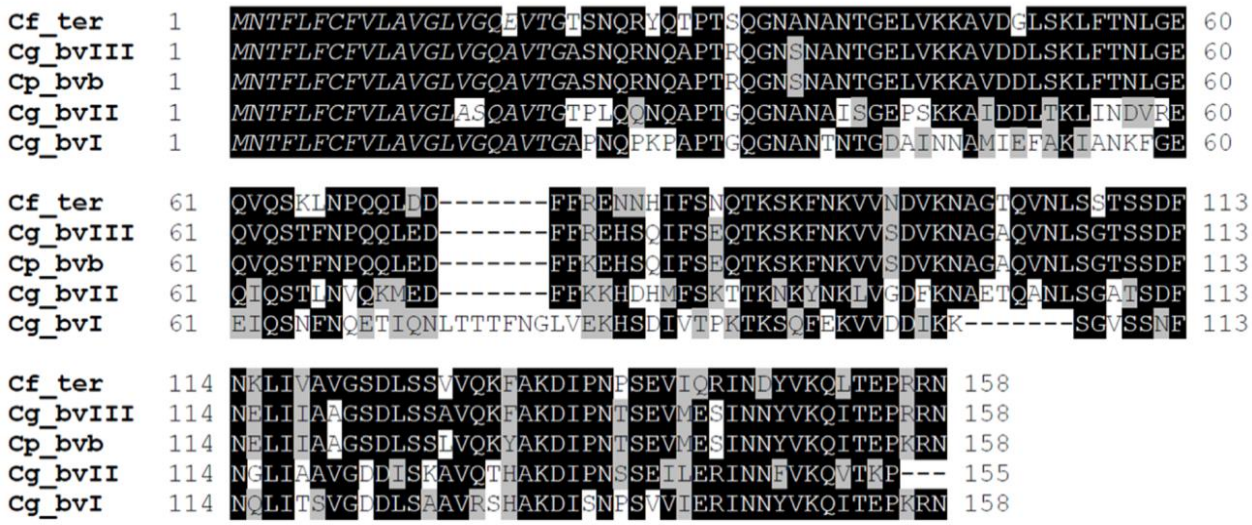

Figure 5. Sequence alignment of Host Translation Inhibitor Factor detected in the teratocytes of *Cotesia flavipes* (CftHTIF) with other HTIFs. CftHTIF was aligned with three HTIFs from *Cotesia congregata* bracovirus (Cg_bvI, Cg_bvII and Cg_bvIII) and one from *Cotesia plutellae* bracovirus (Cp_bvb). Italic letters represent signal peptides. Identical amino acids and conservative replacements are shown in black and gray boxes, respectively. Accession numbers are: Cg_bvI (YP_009665795.1), Cg_bvII (YP_009665794.1) and Cg_bvIIIm (YP_009665793.1), Cp-bvb (ABI16035.1) and Cf_ter (this study).

3.3. Alignment and molecular modeling of a selected defensin expressed by the teratocytes of Cotesia flavipes

The teratocyte-derived defensin shows identity with another insect species in which we selected non-redundant species for analysis (% identity; E value): *C. vestalis* (40.58%; 5^{-09}), *B. tabaci* (40.58%; 1^{-08}), *G. mellonella* (39.13%; 1^{-08}), *L. sinapis* (35.71%; 9^{-07}), *H. armigera* (30.43%; 1^{-05}), *C. virescens* (30.43%; 1^{-05}) and *M. sexta* (33.33%; 1^{-04}). A model generated for the highest expressed defensin peptide, based on the defensing from *Archaeoprepona demophon* (Linnaeus, 1758) (Lepidoptera: Nymphalidae) (Landon

et al. 2004; PDB entry 1P00), presents one α-helix, three β-sheets and three loops, where the α-helix is linked to a β -sheet by two disulfide bridges (Fig.6B).

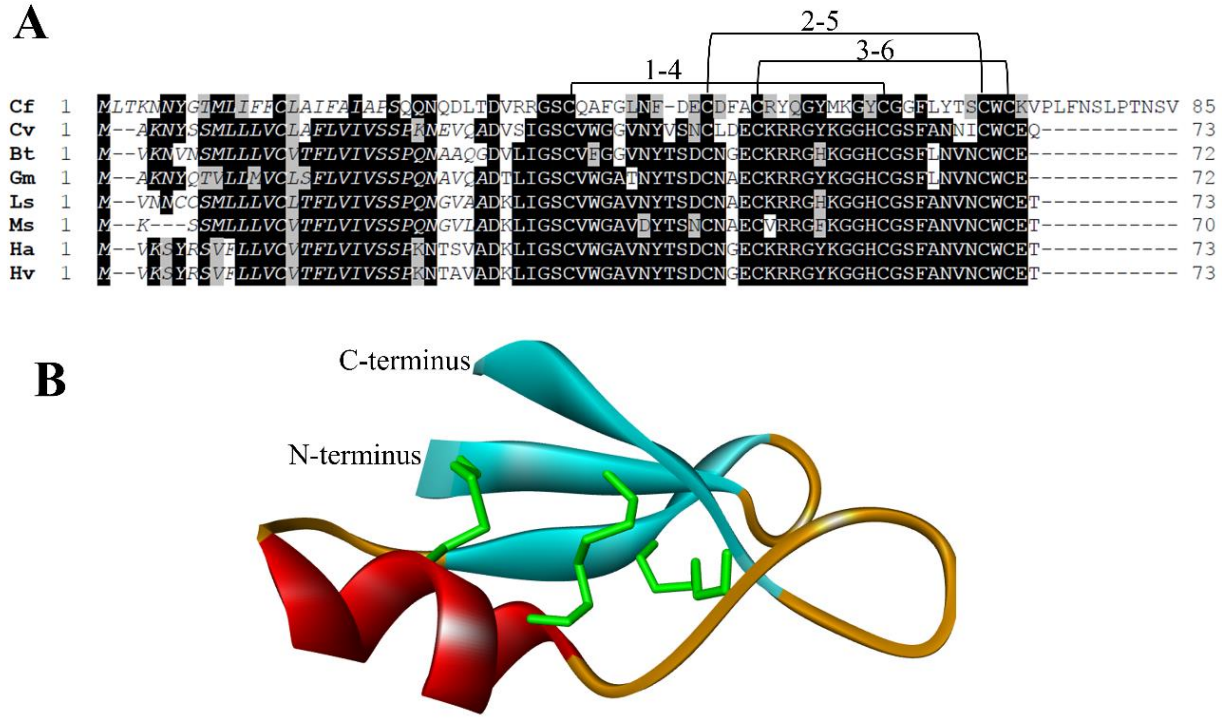

Figure 6: **Structural analysis of a highly expressed antimicrobial defensin from teratocytes of *Cotesia flavipes*.** (A) Sequence comparison of teratocyte-derived defensin from *C. flavipes* with other insect species. Italic letters represent signal peptides. Disulfide bridge connectivities are shown above the sequences. Identical amino acids and conserved replacements are shown in black and gray boxes, respectivelly. Acession numbers are: *Cotesia vestalis* (Cv; AGE89782.1), *Bemisia tabaci* (Bt; XP_018901504.1), *Galleria melonella* (Gm; XP_026765201.1), *Leptidea sinapis* (Ls, VVC93999.1), *Helicoverpa armigera* (Ha; ADR51145.1), *Manduca sexta* (Ms; ADQ00386.1) and *Heliothis virescens* (Hv ACR78445.1). (B) Molecular modelling of a *C. flavipes* teratocyte-derived defensin. The conserved disulfide bridges are shown in green. The α-helix (red) is linked to a β-sheet (blue) by two disulfide bridges, plus a disulfide bond connecting a loop (orange) to another β-sheet. This model fits the best quality parameters among 50 templates searched, confirming the defensin fold.

4. Discussion

In this study, we examined host regulation by the endoparasitoid wasp, *C. flavipes,* over *D. saccharalis* larvae, focusing on cellular components of the immune system of the host and the teratocytes of the parasitoid. In general, we (*i*) observed that parasitism

altered host cellular immune-related factors, (*ii*) identified major secretory products produced by teratocytes, and (*iii*) unraveled the time-course accumulation of different teratocytes-derived peptides in the hemolymph of the host from day 0 (just after parasitoid oviposition) until 10 DAP.

After oviposition into the host haemocoel, the modulation of the host physiology by endoparasitoids aim to avoid the response of the immune system of the host over the eggs of the parasitoid (Ali et al. 2015, Gundersen-Rindal et al. 2013). Following, for appropriate parasitoid larval development, modulations of the host immune system is still required to avoid the cellular or humoral responses such as encapsulation followed by melanization of the parasitoid larvae given by, respectively, the action of hemocytes and the activity of the enzyme PO (Sheehan et al. 2020).

In this scenario, parasitoids like *C. flavipes* are armed with different tools such as venom, calix fluids and symbiotic viruses that are injected during oviposition by the female wasp in the host as well teratocytes and the larvae that develop inside the host (Burke and Strand 2014, Moreau and Asgari 2015, Gao et al. 2016, Lüthi et al. 2020). In a time-course analysis, it is expected that venom, calix fluids, and symbiotic viruses act in the first days of the interaction to protect the egg until larval eclosion. Following larval eclosion, new mechanisms for host regulation may be required and the symbiotic polydnaviruses continues to act in host regulation, but now aided by released teratocytes and the developing larvae (Schafellner et al. 2007, Ali et al. 2015). After parasitoid larval eclosion, the needs of the parasitoid in terms of nutrition will change, and we figure that host regulation during parasitoid larval stage may be different between egg and larval stages of the parasitoid.

In the beginning of the interaction between *C. flavipes* and *D. saccharalis*, we observed a practically instantaneous reduction in host hemocyte viability (0-2 DAP, parasitoid egg stage) just after parasitoid oviposition. We associate this reduction with the action of venom, calix fluids of *C. flavipes* or symbiotic polydnavirus. From 3 DAP on, a significant reduction in total hemocyte counting (THC) was observed. This reduction of THC in the hemolymph may be caused by interferences in host hematopoiesis, the process responsible for production of hemocytes (Teramoto and Tanaka 2004, Hillyer 2016).

The venom of the parasitoid *Pimpla turionellae* (Linnaeus, 1758) (Hymenoptera: Ichneumonidae) was shown to be responsible for the reduction of the number of hemocytes and granulocytes, the cells that guide the encapsulation of foreign bodies, in the host *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) (Er et al. 2010; Hillyer 2016). Additionally, enzymes present in the venom of parasitoids, such as Ras homologous GTPase activating protein (RhoGAP) and sarco/endoplasmic reticulum calcium ATPase (SERCA), are known to interfere in primordial functions of hemocytes, reducing the immune response of hosts (Yan et al. 2016).

Despite the known effects of venom acting on the reduction in THC, there are still, at least, two possibilities in this regulation observed in *C. flavipes-D. saccharalis* interaction: (*i*) the venom of *C. flavipes* act slowly, since reduction in THC started only at 3 DAP or (*ii*) the venom components are not involved in the reduction of THC in *D. saccharalis*. This second hypothesis is suggested after studies that indicated the venom gland as not essential for successful parasitism in polydnaviruses-carrying parasitoids (Dorémus et al. 2013). Complementary studies are required to solve this additional issue raised by our investigation.

Polydnavirus-derived factors are also associated with altered hemocyte behavior, and reduction of THC (Nalini and Kim 2007, Suzuki et al. 2008, Gundersen-Rindal et al. 2013, Kwon and Kim 2008). Using our proteotranscriptomic approach, we detected the presence of eight viral proteins in the earlier DAP (1-4 DAP). So, at least partially, the reduction of THC and hemocytes viability in earlier DAP may be related to bracovirus-derived proteins. Reinforcing this suggestion, at 4 DAP, we detected an EP1-like bracovirus-derived protein, a protein known to be responsible for the reduction of hemocyte population in other insect host (Kwon and Kim, 2008).

The detection of polydnaviruses genes (1-3 DAP) before teratocytes release (4 DAP) using the transcriptome of teratocytes as a reference for the proteomics may be explained by the fact that polydnaviruses use teratocytes as a tissue for gene expression in *D. saccharalis* parasitized by *C. flavipes*. In addition, a HTIF gene from *C. flavipes* is expressed in much increased rates in teratocytes than in hemocytes, fat body and midgut of *D. saccharalis* (Rossi 2012). Lower levels of viral gene expression in teratocytes were

sufficient to include them in our proteotranscriptome dataset. The viral proteins detected before teratocytes release were probably expressed in other tissues like hemocytes.

A reduction in the protein content in larval *D. saccharalis* plasma after parasitism by *C. flavipes* was consistent with previous observations (Salvador and Cônsoli 2008, Passos et al. 2019). The reduction of the total protein content in parasitized larvae is associated with the consumption of polypeptides from the hemolymph of the host by the immature parasitoid (Nakamatsu and Tanaka 2004). The reduction in protein content in the hemolymph of the host may be also associated with a modulation in the metabolism of the host. Interestingly, we detected a polydnavirus-related protein as the highest expressed protein in *C. flavipes* teratocytes that may explain the reduced protein content. This viral protein is a host translation inhibitory factor (CftHTIF), a protein associated with the inhibition of the synthesis of storage proteins in the host (Barandoc and Kim 2009, Barandoc and Kim 2010), which can explain, along with parasitoid consumption and growth, the pronounced reduction in protein content in the hemolymph of the host from 6 DAP on.

The total number and function of the teratocytes vary according species and type of parasitoid and may also vary intraspecifically, depending on the host or environmental conditions (Hotta et al. 2001, Strand 2014). The reduction in teratocytes total count during parasitoid development observed in this work suggest that parasitoid larvae inside the host may be feeding on them (Okuda and Kadono-Okuda 1995). In addition, the occurrence of apoptosis and degradation of teratocytes may also be occuring (De Buron and Beckage 1997). In our time-course experiment, we detected a later accumulation of cysteine-proteases and cystatins, enzymes and inhibitors possibly related with apoptosis, suggesting that a coordinated cell and tissue remodeling may be driven by the parasitoid (Espagne et al. 2005).

In addition to the variation on the teratocyte count, we demonstrated that *C. flavipes* teratocytes also vary in size during the parasitic stage. Generally, teratocytes are indivisible cells, but such difference in diameter may occur due to eventual cellular division of the multinucleated cells into smaller uninucleate cells, as shown for teratocytes of *Encarsia pergandiella* Howard, 1907(Hymenoptera: Aphelinidae) (Mancini et al. 2016). The process of polyploidization, the replication of cellular nuclei without cell division,

directly affects teratocyte size (Mancini et al. 2013, Mancini et al. 2016). Despite the possibility of division, once we observed a substantial decrease in *C. flavipes* teratocytes total count during parasitism, division of *C. flavipes* teratocytes is likely to be minimal. Complementary, the decrease in teratocytes number during parasitism also reinforce the theories that teratocytes are being consumed by parasitoid larvae or that teratocytes apoptosis is happening.

The PO is a humoral complement of the cellular defense that acts in the melanization of foreign bodies such as fungi and bacteria that are nodulated or parasitoids that are encapsulated by hemocytes (Nuñez-Valdez et al. 2019, Strand 2008, Lemaitre and Hoffmann 2007). We detected that *C. flavipes* parasitism reduced PO activity of *D. saccharalis* in the beginning of parasitism (2-3 DAP) and at later phases of the parasitic stage (7-10 DAP). Different host regulation-associated factors from the parasitoid may be reducing PO activity in *D. saccharalis*. Venom, polydnaviruses and teratocytes were already related to the reduction of PO activity of hosts during parasitoid larval development (Zhu et al. 2013, Burke and Strand 2014; Yan et al. 2016; Mahmoud et al. 2011, Gao et al. 2016).

Using our proteotranscritomic analysis, we detected 2 serpins produced by teratocytes from 5 DAP on. Serpins are serine protease inhibitors that interacts with proteases at their flexible and solvent-exposed reactive center loop (Marijanovic et al. 2019). The serpins expressed by teratocytes are suggested to be involved in downregulation of the host PO response by avoiding the activation of pro-phenoloxidase (pro-PO, a catalytically inactive form of phenoloxidase) by serine proteases that are expressed after an infection (Strand 2014, Yang et al. 2017). Serpins are mostly released by teratocytes but also found in the maternal venom. The presence of serpins in the venom may explain the reduced activation of pro-PO before teratocytes release (Ali et al. 2015).

Despite a significant reduction of cellular and humoral components of the host *D. saccharalis* after parasitism by *C. flavipes*, the activity of lysozyme was less impacted in parasitized larvae, being reduced specifically at 5 and from 7-10 DAP, a state of the host that most measured features tended to drastically reduce. Lysozymes are antimicrobial lytic enzymes that catalyze hydrolysis between *N*-acetylmuramic acid and *N*-acetyl-D-

glucosamine units of bacterial cell wall (Nash et al. 2006, Freitak et al. 2007, Hillyer 2016). Our results suggest that the parasitoid *C. flavipes* selectively disables immune defenses that would be negative for parasitoid larval development (*e.g.* hemocytes, PO) keeping the activity of immune components that may avoid the development of microorganisms as competitors of the parasitoid larvae in development inside the host. Moreover, when lysozyme activity and immune responses of the host are knocked-out at 7-10 DAP, teratocytes starts the production of at least 7 antimicrobial peptides.

Confirming our observations, recent findings show that parasitoids invest in the creation of an aseptic environment in the immune depressed host by releasing, especially via teratocytes, several anti-microbial proteins into the host hemolymph (Gao et al. 2016). For instance, we found a highly expressed defensin, previously reported as an important humoral factor released by teratocytes, able to control bacterial and fungal infections (Gao et al. 2016). Insect defensins have a broad-spectrum activity against several Gram-positive and Gram-negative bacteria and fungi (Koehback 2017). An *in silico* analysis of our defensin shows that conserved cysteines are correctly placed, forming a known cysteine-stabilized αβ motif structure (Shafee et al. 2017). Defensins recognize and disrupt bacterial cells by membrane pore formation and cell wall disruption (Nygaard et al. 2012). This is an interesting feature because antimicrobial peptides from parasitoids could be used as templates for the design of new antibiotics with specific modes of action (Shen et al. 2009, Zhu et al. 2011, Moreau and Asgari 2015, Gao et al. 2016, Koehback 2017).

A chitinase and a chitin binding peritrophin were detected at 10 DAP, a similar observation also made by Cônsoli et al. (2005), that detected a chitinase (posteriorly classified as a chitolectin) in the last day of the parasitic stage. The functions of these chitin hydrolyzing or chitin binding proteins are suggested as (*i*) aiding parasitoid larval emergence from the host or (*ii*) act as antimicrobials. Further investigation on the chitin metabolism are necessary to unravel the role of these chitin-related proteins.

Finally, our findings reinforce the importance of teratocytes on parasitism process showing the expression of several molecules with distinct host regulatory functions in this cell-type. Perhaps, immune disruption is the main function, but we also show that the creation of a new aseptic environment is as much important. These results may be the

startup for future projects of the functional and biotechnological use of teratocyte-derived molecules.

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Chapter IV - Venomics and functional analysis of the endoparasitoid *Cotesia flavipes* (Hymenoptera: Braconidae) venom peptides

Abstract

Endoparasitoid wasps use an extensive and complex biochemical arsenal to suppress the normal humoral and cellular immune responses of their host, transforming them into a suitable environment for the development of their eggs and larvae. Venom injected during oviposition is a key component of this arsenal, but the functions of their venom toxins are currently poorly understood. Furthermore, characterization of venom toxins for potential biotechnological use, for example control of agricultural pests, is scarce. *Cotesia flavipes* (Hymenoptera: Braconidae) is a biocontrol agent raised and released extensively in Brazil to control the sugarcane borer *Diatraea saccharalis* (Lepidoptera: Crambidae). The objective of this work was to reveal venom components produced by *Cotesia flavipes* (Hymenoptera: Braconidae) and explore the functionality of a previously unknown highly abundant peptide, H-BCTX-Cf4. Using a combined proteomic/transcriptomic approach, we discovered 38 putative venom toxins including linear and disulfide-rich peptides, enzymes such as hydrolases and trehalase, protease inhibitors, apolipophorins, lipid-binding proteins, and proteins of the odorant binding families. Based on its sequence features and high abundance in venom extract, we synthesized Cf4, a 33 amino acid peptide with three disulfide bonds. The synthetic peptide was shown to modulate *D. saccharalis* hemocyte function (without any effect on phenol oxidase activity), consistent with disruption of the cellular immune response. We also found that feeding leaves coated with Cf4 to neonate *D. saccharalis* resulted in increased mortality and strongly reduced feeding in comparison to caterpillars fed normal leaves, indicating this peptide is a candidate for further investigations on pest control. This study adds to our knowledge of endoparasitoid venoms and their potential use in biotechnology.

Keywords: toxin, peptide toxin, koinobiont, host regulation, biological control, Aculeata

1. Introduction

Parasitoid wasps use other organisms, usually arachnids or insects, as hosts for their offspring's development (Moreau and Asgari 2015, Burke and Strand 2014). This highly successful life strategy has led them to account for an estimated 10–20% of all insect species (Heraty 2009, Yan et al. 2016). Parasitoid wasps have high value as natural and applied biological control agents in crops. For example, about 3.5 million ha of Brazilian crops are treated annually with massive releases of the endoparasitoid *Cotesia flavipes* Cameron (Hymenoptera: Braconidae) for controlling the sugarcane borer *Diatraea saccharalis* Fabricius (Lepidoptera: Crambidae) (Parra and Coelho 2019) (Fig. 1).

Figure 1. *Cotesia flavipes* (Hymenoptera: Braconidae) parasitizing *Diatraea saccharalis* (Lepidoptera: Braconidae).

Parasitoid wasps use a range of strategies to facilitate successful parasitism. The larvae of endoparasitoid koinobionts such as *C. flavipes* develop inside the hemocoel and allow some continued development and growth of the host; unlike idiobiont species that paralyze the host, or ectoparasitoids that occur on the host's outside. Successful endoparasitism depends on establishing a propitious environment for larval development, especially by knocking down the host cellular and humoral immune systems (Fang et al. 2016; Aliet al. 2015; Tan et al. 2018). Since their larvae have a prolonged interaction with the living host, koinobiont parasitoids have the most complex and subtle strategies of host regulation (Pennacchio and Strand 2006). The adaptive tools used by parasitoids to control their host's metabolism, including the immune system, are collectively termed host regulation factors. Endoparasitoids employ a greater range of host regulation factors compared to ectoparasitoids, including venom, polydnaviruses, teratocytes, and the larvae themselves (Strand 2012, Strand 2014, Schafellner et al. 2007).

Endoparasitoid venom is injected into hosts during oviposition and has been proposed to perform several roles including paralysis, immune suppression, and nutrient mobilization (Moreau and Asgari 2015, Becchimanzi et al. 2020, Danneels et al. 2010, Mrinalini and Werren 2015, Siebert et al. 2019). For immune suppression, important targets are host hemocytes and the phenoloxidase (PO) pathway that represent the cellular and humoral immune responses respectively. Both of these are targeted by venom components of the ichneumonid endoparasitoid *Pimpla rufipes* (Miller, 1759) (Hymenoptera: Ichneumonidae) (Asgari et al. 2003, Richards et al. 2008). Other studies have examined the composition of braconid endo- and ectoparasitoids (Zhang et al. 2005, Teng et al. 2016, Wu et al. 2020) including *Cotesia* sp. (Asgari et al. 2003, Teng et al. 2016, Teng et al. 2017, Zhao et al. 2017), but the function of braconid endoparasitoid venom components is less well characterized.

Recent studies have also highlighted the potential of endoparasitoid venom toxins in biotechnology. For example, venom of the endoparasitoid wasp *Aenasius arizonensis* (Girault, 1915) (Hymenoptera: Encyrtidae) has insecticidal activity towards its mealybug host *Phenacoccus solenopsis* Tinsley, 1898 (Hemiptera: Pseudococcidae) (Abbas, 2021). An endonuclease from venom of the endoparasitoid wasp *Pteromalus puparum* (Linnaeus, 1758) (Hymenoptera: Pteromalidae) induces cell death when transfected into

lepidopteran cell lines, one of the few endoparasitoid venom proteins with a known mode of action (Wang et al. 2020).

A precise characterization of the venom components is crucial to understanding the molecular interplay between endoparasites and their hosts. Despite this, and the potential utility of their toxins, the venom composition of the vast majority of endoparasitoid wasps about 20,000 described species remain unrevealed (Pennacchio and Strand 2006, Tang et al. 2019). Combined transcriptomics and proteomics have been successfully used to characterize parasitoid venom (Teng et al. 2017), and high-throughput combined “omics” approaches are likely to yield the composition of venom from diverse parasitoid wasp species in the near future (Becchimanzi et al. 2020).

In this study we applied combined transcriptomics and proteomics to examine the venom composition of *C. flavipes*. Furthermore, aiming to investigate whether *C. flavipes* venom may be biotechnologically explored, we synthesized a putative venom peptide, Cf4, and demonstrate its biological activity on hemocytes and when ingested by neonate *D. saccharalis*. This study provides insights into the composition of *C. flavipes* venom and highlights endoparasitoid venoms as a source of biopesticides and pharmacological agents.

2. Material and Methods

2.1. Insects and venom extraction

Adult *C. flavipes* were kindly donated by a mass breeding biofactory located in São Paulo state, Brazil (21°21′23″S, 48°3′48″W). Before the extraction of glands and venom reservoir, the insects were kept under controlled conditions for 48 h after emergence (25 ± 1ºC; 60 ± 10% RH; 14 h of photophase). For venom apparatus extraction, 200 insects were firstly submerged and kept in anticoagulant buffer (0.098 M NaOH, 0.15 M NaCl, 0.017 M EDTA, 0.046 M citric acid) (Hotta et al. 2001). Using a stereomicroscope, fine forceps, and pins the venom glands and reservoirs were dissected. Subsequently, samples were macerated, centrifuged (14,000g, 4°C, 5 min), and the supernatant was collected and stored at –80°C.

2.2. Transcriptomics

We performed RNA extractions from 4 mg female wasps using TRIZOL reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. Total RNA was resuspended in ultrapure water and quantified using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA, USA). RNA-Seq library construction and sequencing was performed by the Institute for Molecular Biosciences Sequencing Facility at The University of Queensland, Brisbane, Australia. TruSeq libraries with 150 bp inserts were constructed and sequenced in paired-end format according to the manufacturer's instructions using a HiSeq 2500 instrument (Illumina, San Diego, CA, USA). Reads were obtained and were assembled *de novo* using an automated pipeline for trimming, correcting and assembling RNA-Seq reads using fqtrim (Geo Pertea 2018), prinseq (Schmieder and Edwards 2011), and Trinity 2.4.0 (Haas et al. 2013) software packages. Trinity's TransDecoder software was then used to translate open reading frames >90 bp and the resulting amino acid sequences, together with a list of 200 common MS contaminants, were used as a search database for comparison to mass spectra.

2.3. Mass spectrometry

Protein extracted from the parasitoid venom apparatus was submitted to liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). We prepared reduced, alkylated and trypsinized samples by incubating ~100 μg of protein at 37°C for 1 h in reducing/alkylating buffer (1% 2-iodoethanol, 0.25% triethylphosphine, 48.75% acetonitrile, 50 mM ammonium bicarbonate pH 11.0). Subsequently, samples were dried in a vacuum centrifuge (1 h) and resuspended in digestion buffer (40 mM ammonium bicarbonate pH 8.0, 10% acetonitrile) with 20 ng/μL sequencing grade trypsin (Sigma Aldrich, St. Louis, MO, USA) and incubated for 2 h at 37°C. Trypsin was inactivated by addition of 50 μL extraction reagent (50% acetonitrile, 5% formic acid) and dried in speed vacuum again (1 h). Finally, sample was resuspended in 1% formic acid. Non-trypsinized samples were prepared by omitting the digestion step.

MS samples were loaded onto a 150 × 0.1 mm Zorbax 300SB-C_{18} column (Agilent, Santa Clara, CA, USA) on a Shimadzu Nano LC system with the outflow coupled to a

SCIEX 5600 Triple TOF (Framingham, MA, USA) mass spectrometer equipped with a Turbo V ion source. Peptides were eluted using a 70 min gradient of 1–40% solvent B (90% acetonitrile and 0.1% formic acid) in solvent A (0.1% formic acid) at a flow rate of 0.2 mL/min. For MS1 scans, *m/z* was set between 350 and 2200. Precursor ions with *m/z* 350–1500, charge of +2 to +5, and signals with >100 counts/s (excluding isotopes within 2 Da) were selected for fragmentation, and MS2 scans were collected over a range of 80–1500 *m/z*. Scans were obtained with an accumulation time of 250 ms and a cycle of 4 s. The generated mass spectra were compared to the transcriptome library obtained from *C. flavipes* teratocytes with a Paragon 4.0.0.0 algorithm in ProteinPilot 4.0.8085 software (SCIEX, Framingham, MA, USA). Incomplete ORFs were re-analysed by mapping trimmed reads with Geneious software. Final protein annotation was performed using SignalP (Armenteros et al. 2019), BLAST searches (Altschul et al. 1990) with cutoff $E < 1e^{-5}$ against the UniRef90 and SwissProt databases, and HMMER searches (Eddy 2008) against the Pfam database.

To estimate the abundance of each putative venom polypeptide, we used a modified exponential modified protein abundance index (emPAI) (Ishihama et al. 2005) protocol, emPAI65. Mass spectra from reduced, alkylated and trypsinized venom gland extract were compared with a database containing the estimated mature sequences of toxins (*i.e.*, without signal peptides; see Supplementary Data S1) using the Paragon algorithm in ProteinPilot. The proportion of the mature protein detected with 95% confidence (cov95%) was used to calculate $emPAI65 = 6.5^{cov95\%} – 1$ according to an optimized method (Kudlicki 2012).

2.4. Peptide synthesis and purification

Cf4 was produced by solid phase peptide synthesis (SPPS) using a Liberty Blue automated microwave synthesizer (CEM, Charlotte, NC, USA) at 0.1 mmol scale using Fmoc chemistry and Wang-Glu resin. Couplings were performed in dimethylformamide (DMF) using 5 eq of Fmoc-protected amino acid/0.25 M Oxyma Pure/2 M *N*,*N*′-diisopropylcarbodiimide, relative to resin substitution, for 1 min at 105°C. Fmoc was removed by treatment with 25% pyrrolidine/DMF (40 s at 100°C). Side-chain protecting groups used were Arg-Pbf, Lys-Boc, Ser/Tyr-tBu, Asn/His-Trt, and Glu-OtBu. Cleavage

and simultaneous removal of side-chain protecting groups were carried out using a CEM Razor rapid peptide cleavage system for 30–60 min at 40–45°C in cleavage solution (92.5% TFA, 2.5% triisopropylsilane, 2.5% H_2O, 2.5% anisole or 3,6-dioxa-1,8-octanedithiol). Following filtration of cleavage solution, ice-cold diethyl ether was added to precipitate crude peptides. Crude peptides were washed with diethyl ether and centrifuged three times (6000g, 5 min), then resuspended in 0.1% TFA/50% ACN/H_2O, filtered, and lyophilized. Crude Cf4 was purified by preparative RP-HPLC using a Vydac C_{18} column and a linear gradient of 0–60% solvent B (0.043% TFA, 90% acetonitrile (ACN)) in solvent A (0.05% TFA in water) over 50 min. The crude Cf4 products were first treated with dithiothreitol (DTT) for 2 h (peptide dissolved at 1 mg/mL in 100 mM DTT in 0.1 M phosphate buffer, pH 4) and then purified using RP-HPLC. The expected mass was confirmed using electro spray ionization mass spectrometry (ESI-MS) and matrix-sssisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) using an AB SCIEX TOF/TOF™ 5800 spectrometer. For MALDI-TOF-MS, 0.4 µL sample mixed with 0.5 µL of matrix [5 mg/mL α-cyano-4-hydroxycinnamic acid (α-CHCA), 70% ACN and 0.1% formic acid (FA)] was spotted, and mass spectra were acquired using laser intensity of 3600–4300, mass detection range 1500–9000 Da, and focus mass 3500–4000 Da. The RP-HPLC fraction containing the synthetic venom peptide was lyophilized and resuspended in PBS. Peptide concentration was estimated from A_{280} measured on a Nanodrop spectrophotometer using the predicted extinction coefficient calculated using ProtParam (https://web.expasy.org/protparam/). The peptide was folded in a redox buffer (15 µM in 100 mM Tris-HCl pH 8.0, 1 mM reduced glutathione, 1 mM oxidized glutathione) for 24 h at a peptide concentration of 0.1 mg/mL. Following oxidation, the purity and mass of Cf4 were monitoried using RP-HPLC, MALDI-TOF-MS and ESI-MS.

2.5. Enzymatic activity: Phenol oxidase and lytic activity assays

All assays were performed with three concentrations of peptide, 10, 1 and 0.1 µg. To prepare the cellular *in vitro* PO assay, 100 µL of hemolymph from 5th instar *D. saccharalis* (~3 insects) was withdrawn, centrifuged (1,000, 5 min, 4ºC), the supernatant collected, and the cell pellet was washed twice and resuspended with 100 mL PBS. The cell suspension was transferred to a 24 well plate followed by the addition of the peptide.

After 24 h, cell viability was estimated by Neubauer Chamber (HBG® 9020-01) using Trypan Blue cell viability reagent. Finally, 2 µL of cell suspension ($5x10^5$ cells/mL^{-1}) was transferred to a 96-well plate and mixed with 198 mL of PO substrate buffer (5 mM L-DOPA, 10 mM sodium cacodylate, 5 mM $CaCl_2$ pH 7.0). For the cell-free PO *in vivo* assay, peptide was injected into a 5th instar larva, and hemolymph was collected after 24 h and centrifuged (1,000g, 5 min, 4ºC), supernatant collected, and cell pellet discarded. Subsequently, 2 µL of cell free hemolymph was transferred to a 96-well plate and mixed with 198 mL^{-1} of the PO substrate buffer.

PO activity was determined by measuring the absorbance at 490 nm in 40 s intervals for 120 min using a Multiskan Sky Microplate Spectrophotometer (Thermo Fisher, Waltham, MA, USA) and SkanIt software v.6.1. For *in vivo* PO activity we used the automated script *PO-CALC,* the R-based optimized method by Kohlmeier et al. (2015) that considers different types of noises and reduces inconsistences. For *in vitro* activity we used the absorbance linear growing ($R^2 \geq 0.99$) to calculate PO activity. We considered one unit of PO (U) as the amount of enzyme necessary to increase 0.001 units of absorbance at 490 nm per minute. Complementarily, MT_{50} values (mean effective time in seconds for PO substrate melanization) were calculated with 95% confidence and transformed into LogX using absorbance reads from every 40 sec during 120 min. To plot and analyze the curves we used GraphPad Prism v.9 software (GraphPad Software Inc. La Jolla, Ca) and the accuracy of the data was evaluated based on R^2 values.

For lysozyme assays, lyophilized *Micrococcus lysodeikticus* cells (Sigma Chemicals) were suspended in potassium phosphate buffer (0.05 M; pH 6.4) and 291 µL of this suspension was mixed with 9 µL of cell free hemolymph from 5th instar *D. saccharalis* in a 96-wells plate. The plate was placed in the spectrophotometer and absorbance was continuously measured at 450 nm in 40 s intervals for 120 min. One unit of lysozyme (U) was defined as the amount of enzyme necessary to decrease 0.0001 units of absorbance at 450 nm per minute.

2.6. Hemocyte behavior assessment: Adherence capability and encapsulation

To assess hemocyte adhesion, 10 µL of hemolymph extracted from *D. saccharalis* larvae after 24 h injection of 10, 1 and 0.1 µg of Cf4 were placed in glass slides and

incubated at room temperature for 30 min. The drop of hemolymph was gently washed with PBS to remove debris and unadhered cells. Cells were then fixed with 5% methanol in anticoagulant solution for 5 min (Manachini et al. 2011), stained with Giemsia for 10 min, and washed and covered with a clean coverslip. Slides were photographed under phase contrast microscopy (Zeiss® Axio Imager A2) and the percent of total area covered by cells or clusters of cells adhered to the glass slides was measured using ImageJ software.

To assess *in vitro* cell modulation capability in response to Cf4, 20 µL of hemolymph from 5th instar *D. saccharalis* larvae was mixed with 40 µL of InsectXpress™ (Lonza®) culture medium in an 0.5 mL tube. Next, 20 µL of 0.1% w/v A-25 Sephadex beads and Cf4 (10, 1 and 0.1µg) were added and homogenized. To keep beads in contact with the hemocytes, samples were shaken at room temperature and 100 rpm for 2 hr. Beads were assigned to five classes (I–V) according to the thickness of the hemocyte capsule that we observed and recorded under the phase contrast microscope as previously described (Zhang et al. 2005, Wu et al. 2008, Teng et al. 2016, Li et al. 2018) with adaptions. Briefly, class **I** are the beads with at most ten hemocytes attached; class **II** are the beads with 10-50 hemocytes attached; class **III** are the beads with more than 50 hemocytes attached and mostly covered with a layer of three cells; class **IV** are beads covered with more than 3 layers of cells thinner than the bead diameter; class **V** are beads covered with a layer of cells larger than the bead diameter (Suppl. Fig. 2). The encapsulation index (EI) was calculated by the formula EI= [(Σ defined classes/ Σ number of defined beads) *100]/5.

2.7. Effect of oral ingestion of Cf4

In order to investigate if ingestion of Cf4 can be harmful to *D. saccharalis*, sugarcane leaf discs (IAC95-5000 variety, 3.14 cm^2 area) were covered with ~40 µL of 100 µg mL^{-1} Cf4 or water (negative control) using a brush. Leaves were allowed to superficially dry for 10 min and then placed in a 24-wells plate containing 1 mL of agar at the bottom (1%). Larval viability was assessed after three days by touching larvae with a soft brush, with those that did not react considered as dead. Leaf consumption by

neonates was quantified by collecting the leaf disks after three days of larval feeding, scanning them, and measuring the consumed area with ImageJ software.

2.8. Data processing

For all assays, experiments were performed in a completely randomized design and numerical data were analyzed for normality by Shapiro–Wilk test, homoscedasticity by Bartlett's test. When these assumptions were met, we performed ANOVA and, when needed, the means were compared by Tukey or t test ($P < 0.05$). Data were analyzed and plotted using Graph Prism Pad software v.9.0.

3. Results

3.1. Venomics of Cotesia flavipes

To examine the composition of *C. flavipes* venom, we used a combined transcriptomic and proteomic approach. Due to the small size of adults (~2 mm), we did not attempt to produce a venom gland transcriptome and instead produced a whole-body transcriptome of adult females that should nevertheless contain transcripts that encode venom proteins. To identify venom proteins, we compared tandem mass spectra obtained from dissected venom gland protein extract with open reading frames from the whole-body transcriptome, resulting in 218 protein identifications from the trypsin-digested sample and 9 from undigested samples. Since this dataset contained both redundant protein sequences and numerous proteins likely to act in intracellular housekeeping, we further filtered sequences based on (*i*) redundancy as judged by ProteinPilot Unused and Total Scores; (*ii*) presence of a signal peptide; (*iii*) presence of a stop codon; (*iv*) disqualification of sequences with endoplasmic reticulum retention signals; and (*v*) disqualification of sequences with established non-venom extracellular function (*e.g.*, cuticular proteins) (*vi*) sequences obtained from LC-MS/MS from *C. flavipes* carcass (without venom apparatus). In total, we found 38 putative venom components (Table 1, that were annotated using the SignalP, BLAST, and HMMER algorithms, and named according to rational nomenclature guidelines suggested by King et al. 2008). According to these guidelines, each putative toxin is named in the form U-BCTX-Cf1, in which the prefix U- (=unknown) is a placeholder for pharmacological activity, BCTX stands for

bracotoxin, Cf stands for *C. flavipes*, with a numerical identifier suffix. We also estimated the abundance of each putative toxin in the venom gland extract using a modified exponentially modulated protein abundance index (emPAI). Table 1 summarizes the final list of 38 putative venom proteins and peptides. We also assigned a possible physiological role for each polypeptide in the context of parasitism (Fig. 2C, D), though we note that these are speculative and there is potential for molecules such as peptides and enzymes to have diverse roles in modulation of neural, immune, metabolic, and other activity. We found a statistically significant positive correlation between transcript abundance and protein abundance with a Pearson correlation of 0.3941 ($R^{2=}$ 0.1553; P= 0.0143; Fig. 2E).

In any case, our finding that peptides are the most abundant class of putative venom toxins (estimated 46.2% molar abundance of proteinaceous venom toxins) suggests they are important drivers of venom function, and ideal candidates for subsequent bioassays. The most abundantly detected putative toxins are linear peptides, which are important components of aculeate wasp venoms (Moreau and Asgari 2015) and disulfide-rich peptides that are important components of many arthropod venoms (King and Hardy 2013), both of which are classes that often act in modulation of neural activity. Some peptides such as Cf4 (U-BCTX-Cf4), Cf16, and Cf22 have the cysteine scaffold –C–C–CC–C–C– (dash representing loop of non-Cys residues) typical of peptides that fold into the inhibitor cystine knot (ICK) structure that is common in invertebrate venoms. The latter two probably have an extended C-terminal tail, and Cf22 is a close homologue ($E < 10^{7}$) of ω-conotoxin-like protein 1 from the European honeybee *Apis mellifera*. Aside from peptides, another protein that may modulate neural activity is a homologue of the *Drosophila* protein quiver, a small disulfide-rich protein that modulates voltage-gated potassium channels and acetylcholine-gated channels (Wu et al. 2016).

Many of the proteins identified have homology to known proteins reported to interact with the insect cellular and humoral immune systems, including serpins (inhibit PO cascade; Yan et al. 2017), superoxide dismutase (suppresses melanization; Colinet et al. 2011), a C-type lectin (helps eggs avoid encapsulation; Lee et al. 2008), an M17 family aminopeptidase (antimicrobial effects; Dani et al. 2003), a von Willebrand factor domain protein (reported to modulate encapsulation; Arai et al. 2013), a homologue of the interferon-like '27 kDa haemolymph protein' of the greater wax moth *Galleria*

mellonella (modulates PO cascade; Park et al. 2005), and transferrin (antibacterial properties; Yoshiga et al. 1997). Other putative toxins were identified from families that are involved in lipid recognition and transport and are reported to have additional key roles regulating the immune system. These include apolipophorin-III, which detects pathogens, mediates immune responses, and modulates hemocyte behaviour (Wen et al. 2016); prosaposin, the precursor of the saposins that mediate sphingolipid digestion and modulate multiple aspects of the vertebrate immune system (Darmoise et al. 2010); and two proteins (one of which is a homologue of the Niemann Pick disease related C2 protein) that contain an ML domain (Myeloid differentiation factor 2 Lipid recognition domain), which has been reported to function in the recognition of lipids, especially those of pathogens. We hypothesize these functions either in immune modulation or interference with host lipid nutrition.

Several enzymes were identified, represented by the proteases similar to serine carboxypeptidase (S10), papain (C1), and leucine aminopeptidase (M17) families. Trehalase, which catabolizes the main sugar in insect haemolymph (trehalose) may interfere with host nutrition, and a peptidyl-prolyl cis/trans isomerase which might have a role in toxin folding. Several proteins related to those that bind pheromones and odorants in the olfactory system (Pfam PBP/GOBP and OS-D families) were detected, which we hypothesize may bind other ligands in the context of parasitism. A secreted version of the normally intracellular neurofilament heavy chain was also detected but is of unknown function, along with eight other uncharacterized proteins, some of which were highly abundant, suggesting an important but currently undescribed functional role in venom.

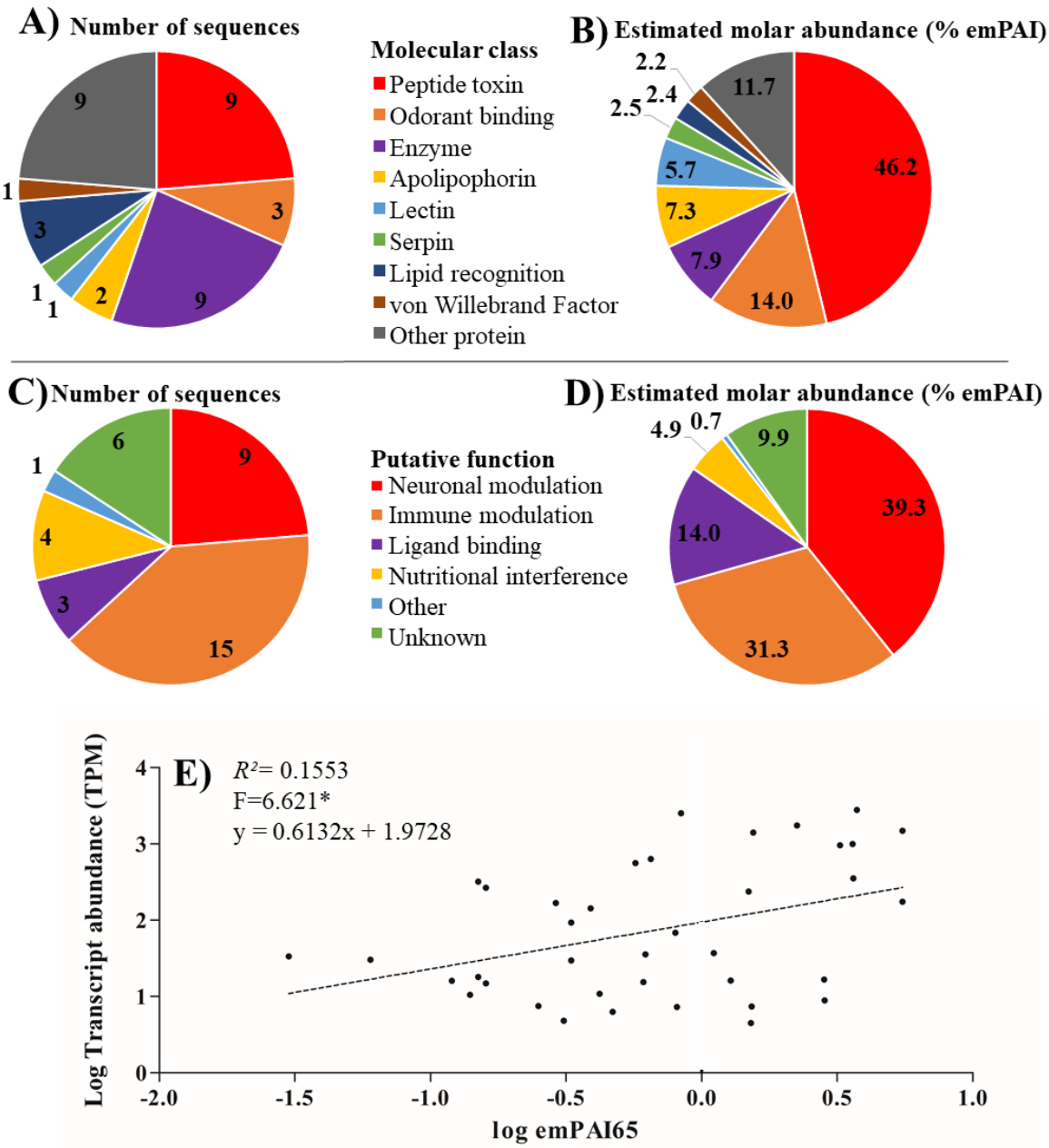

Figure 2. **Identification of putative *Cotesia flavipes* venom proteinaceous components**. (A) Total number of sequences and (B) Estimated molar abundance in venom of each class of proteins identified. (C) Total number of sequences and (D) Estimated molar abundance in venom of proteins according their putative functional roles. (E) Correlation of emPAI65 and transcriptome abundance of identified proteins.

Table 1. Putative venom toxins of *Cotesia flavipes*.

Name	Mature number Cys residues	Mature length (amino acids)	Molar proportion of venom (%)[a]	Molecular class	Putative function	Blast against GenBank nr database top hit (E-value; %ID; accession; species)
U-BCTX-Cf1	0	11	10.95	Peptide toxin	Neuronal modulation	-
U-BCTX-Cf2	0	71	10.95	Peptide toxin	Neuronal modulation	-
U-BCTX-Cf3	0	71	7.44	Peptide toxin	Neuronal modulation	-
H[b]-BCTX-Cf4	6	33	7.22	Peptide toxin (disulfide-rich)	Immune modulation[b]	-
U-BCTX-Cf5	0	173	7.16	Apolipophorin-III	Immune modulation	$7e^{-130}$; 97.91; QBB01877.1; *Cotesia chilonis*
U-BCTX-Cf6	6	118	6.44	Odorant binding	Ligand binding	$4e^{-90}$; 99.26; QBB02065.1; *Cotesia chilonis*
U-BCTX-Cf7	11	249	5.66	Lectin	Immune modulation	$5e^{-118}$; 64.94; XP_008554386.1; *Microplitis demolitor*
U-BCTX-Cf8	6	173	5.63	Other protein	Unknown	-
U-BCTX-Cf9	6	119	4.49	Odorant binding	Ligand binding	$1e^{-96}$; 99.28; QBB02090.1; *Cotesia chilonis*
U-BCTX-Cf10	4	110	3.08	Odorant binding	Ligand binding	$7e^{-85}$; 98.41; QBB01406.1; *Cotesia chilonis*
U-BCTX-Cf11	1	394	3.04	S10 serine carboxypeptidase	Nutritional interference	0; 97.11; APD15616.1; *Cotesia chilonis*
U-BCTX-Cf12	8	77	3.02	Peptide toxin (disulfide-rich)	Neuronal modulation	$4e^{-59}$; 92.63; QBB01483.1; *Cotesia chilonis*
U-BCTX-Cf13	12	76	2.96	Peptide toxin (disulfide-rich)	Neuronal modulation	$1e^{-62}$; 95.92; APD15637.1; *Cotesia chilonis*
U-BCTX-Cf14	2	387	2.54	Serpin	Immune modulation	0; 92.23; APD76157.1; *Cotesia vestalis*
U-BCTX-Cf15	9	128	2.21	von Willebrand Factor	Immune modulation	$1e^{-98}$; 99.31; QBB01606.1; *Cotesia chilonis*
U-BCTX-Cf16	6	49	1.68	Peptide toxin (disulfide-rich)	Neuronal modulation	-
U-BCTX-Cf17	6	408	1.61	Other protein	Unknown	0; 96.72; QBB01797.1; *Cotesia chilonis*
U-BCTX-Cf18	6	130	1.60	ML[c] domain, NPC2[d] homologue	Immune modulation	$2e^{-102}$; 97.97; QBB01740.1; *Cotesia chilonis*
U-BCTX-Cf19	7	327	1.30	C1 cysteine protease	Nutritional interference	0; 99.71; QBB01825.1; *Cotesia chilonis*
U-BCTX-Cf20	7	519	1.23	M17 aminopeptidase	Immune modulation	0; 99.44; QBB01674.1; *Cotesia chilonis*
U-BCTX-Cf21	6	278	1.22	Other protein	Unknown	0; 90.94; QBB01472.1; *Cotesia chilonis*
U-BCTX-Cf22	6	62	1.14	Peptide toxin (disulfide-rich)	Neuronal modulation	$1e^{-52}$;100.00; QBB01511.1; *Cotesia chilonis*
U-BCTX-Cf23	12	281	0.94	Other protein	Immune modulation	0; 97.99; QBB01702.1; *Cotesia chilonis*
U-BCTX-Cf24	1	58	0.84	Peptide toxin	Neuronal modulation	-
U-BCTX-Cf25	8	137	0.77	ML[c] domain	Immune modulation	$2e^{-112}$; 98.71; QBB01364.1; *Cotesia chilonis*
U-BCTX-Cf26	1	188	0.67	PPI[e]	Protein folding	$1e^{-121}$; 83.41; XP_008558950.1; *Microplitis demolitor*
U-BCTX-Cf27	24	690	0.65	Transferrin	Immune modulation	0; 84.23; XP_014300087.1; *Microplitis demolitor*
U-BCTX-Cf28	10	302	0.61	Other protein	Unknown	$6e^{-113}$; 56.43; XP_014300808.1; *Microplitis demolitor*
U-BCTX-Cf29	2	156	0.57	Superoxide dismutase	Immune modulation	$6e^{-119}$; 98.84; QBB01726.1; *Cotesia chilonis*
U-BCTX-Cf30	0	253	0.49	Other protein	Unknown	$1e^{-172}$; 100.00; QBB01432.1; *Cotesia chilonis*
U-BCTX-Cf31	2	474	0.31	Spermine oxidase	Immune modulation	0; 98.38; QBB01752.1; *Cotesia chilonis*
U-BCTX-Cf32	4	415	0.31	Chitinase	Immune modulation	0; 99.77; QBB01968.1; *Cotesia chilonis*
U-BCTX-Cf33	10	133	0.30	Drosophila Quiver homologue	Neuronal modulation	$3e^{-99}$; 85.90; XP_008552222.1; *Microplitis demolitor*
U-BCTX-Cf34	0	459	0.30	Other protein	Unknown	$4e^{-116}$; 62.50; XP_008556008.1; *Microplitis demolitor*
U-BCTX-Cf35	2	549	0.28	Trehalase	Nutritional interference	0; 99.65; QBB01393.1; *Cotesia chilonis*
U-BCTX-Cf36	11	550	0.24	C1 cysteine protease	Nutritional interference	0; 99.82; QBB01621.1; *Cotesia chilonis*
U-BCTX-Cf37	35	3369	0.11	Apolipophorin	Immune modulation	0; 99.15; QBB01949.1; *Cotesia chilonis*
U-BCTX-Cf38	46	837	0.06	Lipid recognition	Immune supressive	0; 80.86; XP_014296735.1; *Microplitis demolitor*

[a]Normalized emPAI metric; [b]Results of this study; [c]Myeloid differentiation factor 2 related lipid recognition domain; [d]Niemann-Pick disease related protein C2; [e]Peptidyl prolyl cis-trans isomerase.

3.2. Synthesis and characterization of U-BCTX-Cf4

From the putative venom toxins of *C. flavipes*, we selected Cf4 for further investigation due to its (*i*) high abundance in venom (according to the emPAI65 metric), (*ii*) presence of six cysteines, and (*iii*) short size (33 amino acid residues) feasible for solid phase synthesis (Upert et al., 2014). Aside from its ICK-like cysteine scaffold, the peptide Cf4 is an unknown molecule, without homology to peptides in other in any of the databases we searched.

After cleavage from the resin and semipreparative reverse phase high performance liquid chromatography (RP-HPLC, Fig. 3A), the main component of the largest peak of the chromatogram was found to have mass closely matching the predicted mass of the reduced amino acid sequence (Fig. 3B,C) by matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF) and electrospray ionization (ESI)-MS. This synthetic peptide was used to investigate the influence of Cf4 on PO activity, cellular and humoral immunity, and caterpillar feeding.

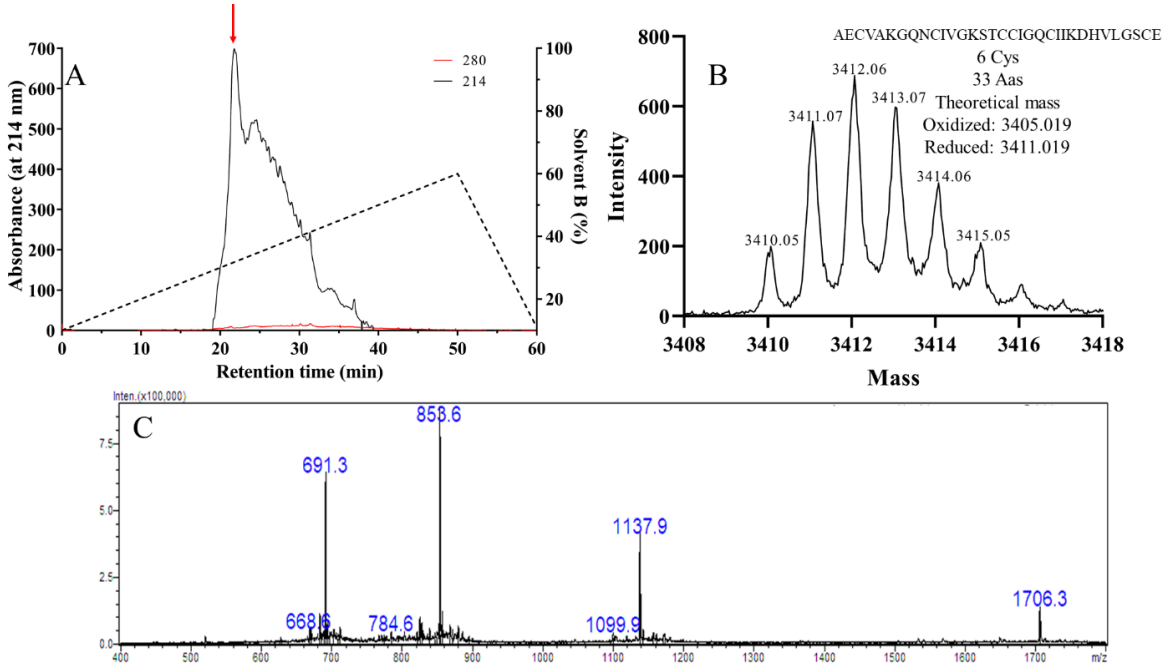

Figure 3. Synthesis of Cf4 peptide. **A**-Semipreparative HPLC trace note: red arrow indicates folded peak. **B**- MALDI-TOF mass spectrum of purified peptide prior to refolding and disulfide bond formation. The measured monoisotopic $M+H^+$ mass, 3410.05 Da, is within the experimental error for this instrument (0.5 Da) from the expected mass, 3409.57 . **C-** ESI mass spectrum for purified peptide prior to refolding. Each peak observed is within the experimental error for this instrument (0.2 Da) from the expected values: $M+2H^+$ =1706.51, $M+3H^+$ = 1138.01, $M+4^+$ = 853.75, $M+ACN+5H^+$ = 691.41.

3.3. Cellular behavior

To test the ability of Cf4 to modulate cellular immunity mechanisms, we injected synthetic peptide in *D. saccharalis* and quantified hemocyte encapsulation of glass beads (see Materials and Methods 4.6 and Supplementary Fig. S1) and hemocyte adhesion to glass slides. When 10 µg of Cf4 was injected into *D. saccharalis* 5th instar larvae, we found that it decreased 1.6-fold the hemocyte encapsulation index ($F_{3,36}$= 9.573; P<0.0001) (Fig. 4A). On the other hand, this same treatment also increased 1.6-fold the hemocyte adhesion capability ($F_{3,28}$= 9.425; P=0.0002) (Fig. 4B). Despite this representing a higher dose than that naturally injected by the female wasp, our results strongly suggest that Cf4 has an important role on hemocyte dysfunction to facilitate successful offspring settlement into the host hemocoel.

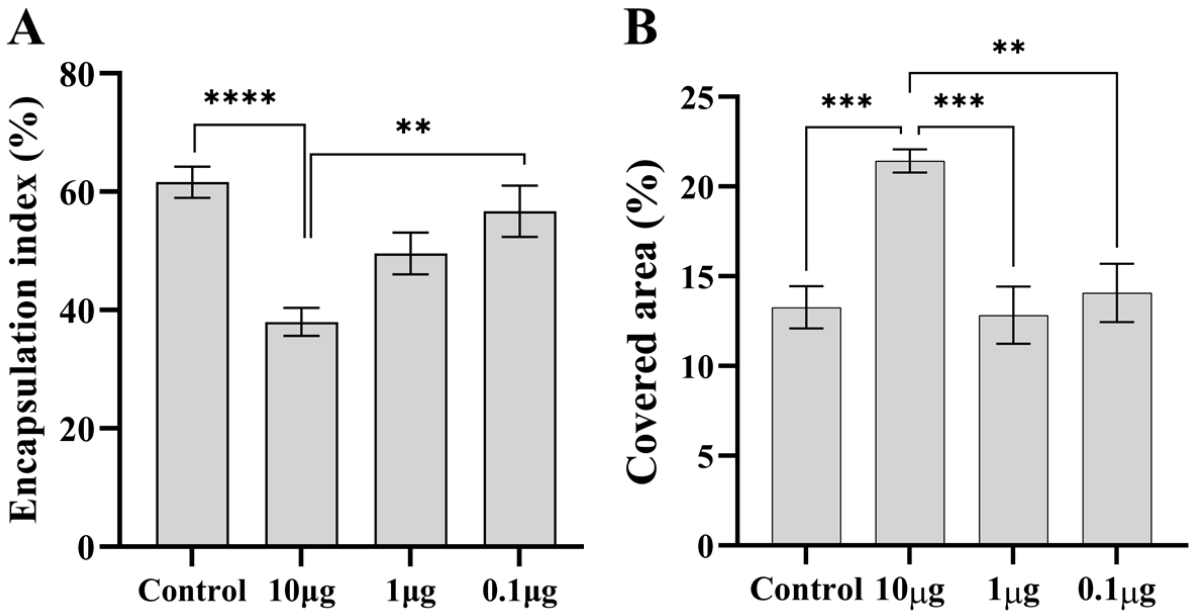

Figure 4. Influence of Cf4 on *Diatraea saccharalis* hemocyte behavior. A- *In vitro* encapsulation index. **B**- Adherence capability (%) of hemocytes quantified by the area of hemocytes adhered to a glass surface (covered area). Bars represent means ± standard errors. Asterisks above the bars indicate significant differences (*P < 0.05, **P < 0.01, ***P<0.001, ****P<0.0001, Tukey test).

3.4. Influence of Cf4 on humoral innate immunity

For the melanization degree analysis, The MT_{50} (LogX) of the treatment with 10 µg was 3.733, slightly higher than the control (Fig. 5A; Table 2). PO activity of soluble ($F_{3,28}$=2.292; P=0.0999; Fig. 5B) and cellular components ($F_{3,31}$=0.6909; P=0.5646;

Fig.5C) of hemolymph were not significantly affected by Cf4. Additionally, the lytic activity of hemolymph is not impaired by this peptide ($F_{3,28}$=0.1842; P=0.9062; Fig. 5D). In summary, these results show that most likely Cf4 is a venom component interferes with cellular but not humoral responses of the host. To reflect this activity, we hereafter refer to H-BCTX-Cf4, with the H-prefix indicating activity on insect hemocytes.

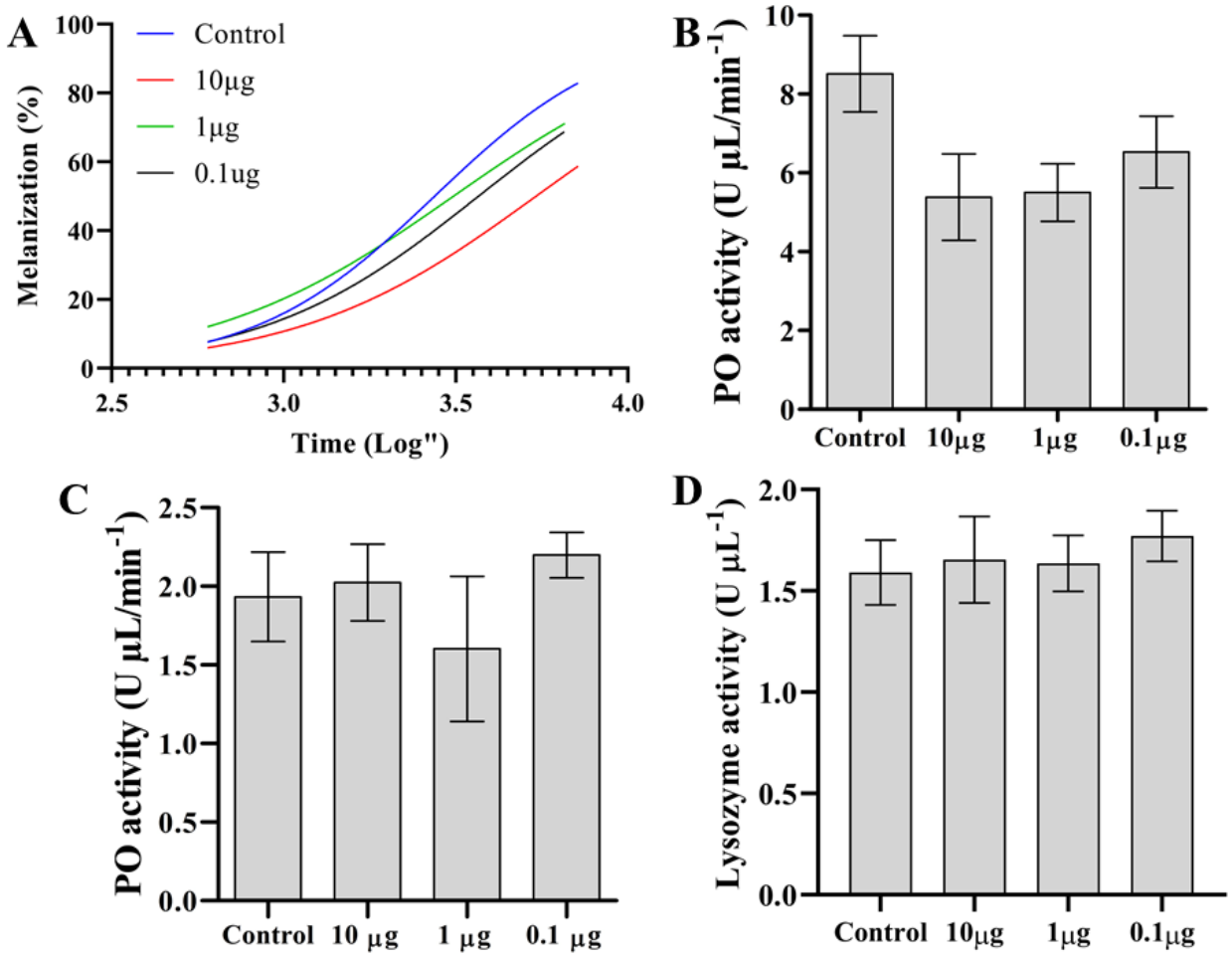

Figure 5. Humoral response facing Cf4, a peptide from *Cotesia flavipes* venom, in different concentrations. A- Melanization sigmoid response for 120 min (Time = Log"). **B-** Phenol oxidase (PO) activity (U μL^{-1}) in the hemolymph plasma of insects injected with Cf4, after 24 h. **C-** Phenol oxidase (PO) activity (U μL^{-1}) of *in vitro* assay with hemocytes in PBS with Cf4 during 24 h. **D-** Lysozyme activity (U μL^{-1}) in the hemolymph plasma of insects injected with Cf4, after 24 h. Bars represent means ± standard errors.

Table 2- Melanization sigmoid curve parameters of Diatraea saccharalis hemolymph with varying amounts of injected Cf4, a peptide from *Cotesia flavipes* venom.

	Control	10 µg	1 µg	0.1 µg
$logMT_{50}$	3.439	3.733	3.493	3.566
95%IC	3.387–3.485	3.663–3.830	3.413–3.568	3.515–3.617
R^2	0.6543	0.3587	0.4006	0.5688

Data are given as median response values together with the 95% confidence interval ($logMT_{50}$).
R^2 represents the accuracy of data fitting to the sigmoid curve.

3.4. Insecticidal potential of Cf4

To test if Cf4 has potential as a bioinsecticide, we tested the effect of leaves with or without a covering of Cf4 to neonates *D. saccharalis*. Our data show that the ingestion of leaves covered with the Cf4 results in a slight reduction in viability of neonates of *D. saccharalis* (t=3.712; df=10 P=0.004; Fig.6A). Furthermore, a more pronounced reduction was observed for food consumption when leaves were coated with Cf4 (t=15.65; df= 10 t=P<0.0001; Fig. 6B).

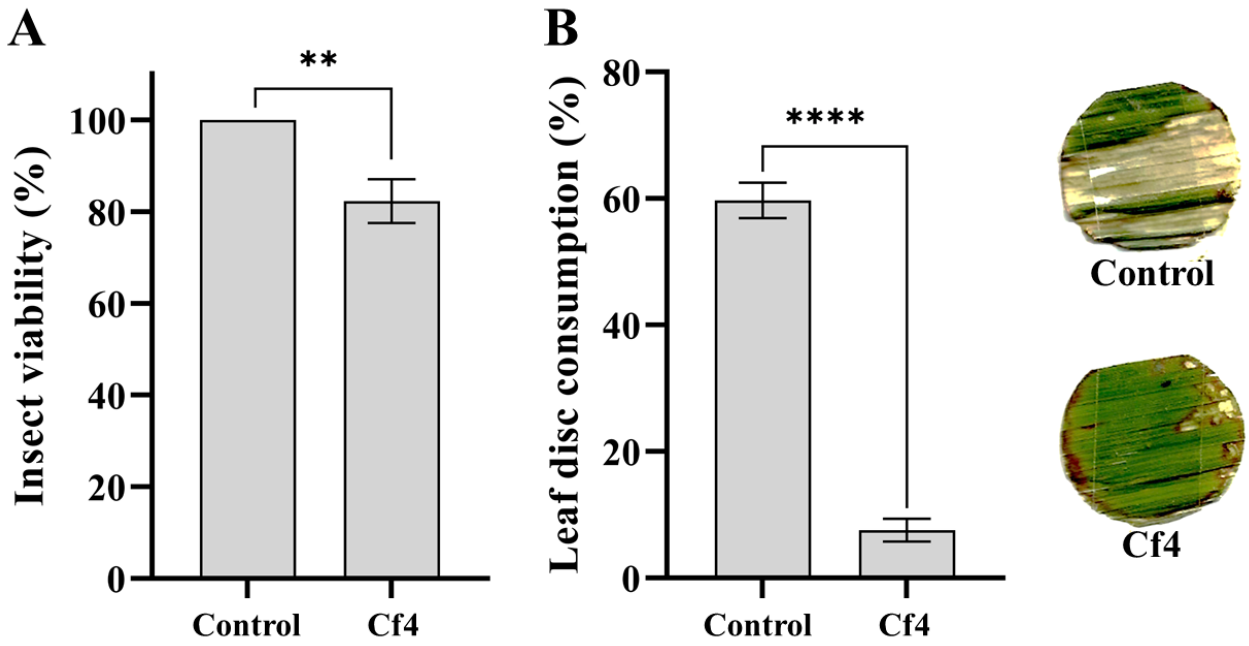

Fig. 6. Insect viability and leaf ingestion by *Diatraea saccharalis* neonates fed with sugarcane leaves covered by 100 µg mL^{-1} Cf4. A- Neonate viability when fed with sugar cane leaves covered with Cf4 compared to normal leaves. **B-** Percentage of leaf disc area consumed in three days by the *D. saccharalis* neonates. The bars represent ± standard error of the means. Asterisks, if present, above the bars indicate significant differences (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, *t* test).

4. Discussion

In this study, we used proteomics and transcriptomics to investigate the venom composition of the endoparasitoid wasp *C. flavipes*, an important biocontrol agent currently used on a massive scale in Brazil. The identified putative venom toxins include numerous and abundant venom peptides, which have previously received little attention in endoparasitoid venoms. We synthesized one of these putative toxins, H-BCTX-Cf4, and characterized it functionally, showing that it changes hemocyte behavior consistent with the major role for venom in immune suppression during parasitism. When applied orally, it inhibited larval viability and feeding, suggesting it may have potential as bioinsecticide.

A fundamental difficulty in venomics of parasitoid wasps such as Cotesia is their small size, which makes it difficult to either dissect sufficient tissue for venom gland cDNA extraction, or obtain venom expelled from the ovipositor, as opposed to dissected from the venom gland. Previous authors using omics to study *Cotesia* venoms have used transcriptomics of venom glands (Zhao et al. 2017) or combined transcriptomics of venom glands and proteomics of dissected venom (Teng et al. 2017).

We did not use a venom gland transcriptome and instead relied on a whole-body transcriptome of female wasps. Disadvantages of this method are that transcript abundance (alone, or in comparison to non-venom tissues) cannot be used to infer a role as a venom toxin, and sequencing depth will be less overall for venom toxins, whose sequences may therefore be incomplete. Nevertheless, we identified 38 amino acid sequences that are complete judging from the presence of a secretion signal peptide and stop codon and match to previous results (Zhao et al. 2017, Teng et al. 2017). Using proteomic spectra from dissected venom instead of 'stung' venom from the tip of the ovipositor, may leads to the identification of non-venom cellular proteins (Walker et al. 2020), but considering that we removed the background signals (from wasps without venom apparatus) and house keeping sequences, we consider our results reliable.

Compared to these previous studies, we undertook additional filtering of sequences to increase the probability of the identified proteins representing venom toxins, including removing sequences without signal sequences and that possessed ER retention signals. We therefore believe most of the putative venom toxins identified likely function

as venom toxins, although we acknowledge that our *C. flavipes* analysis may have led to some false positives as well as false negatives. The assigned putative functions for the identified proteins in immune suppression, nutritional interference, and neural modulation, though these suggestions remain to be tested functionally.

4.1. Immune suppressive proteins

Parasitoid venoms have been reported as important immune disruptors (Qian et al. 2017, Liu et al. 2018, Wu et al. 2020). For example, venom of the ectoparasitoid *Scleroderma guani* (Hymenoptera: Bethylidae) changes hemocyte behavior in *Tenebrio molitor* (Coleoptera: Tenebrionidae) immatures (Li et al. 2018). Similarly, venom of the ectoparasitoid *P. puparum*, decreased encapsulation by *Pieris rapae* (Linnaeus, 1758) (Lepidoptera Pieridae), likely due to the activity of a 24 kDa venom toxin (Wu et al. 2008). In our study, we found that Cf4, a peptide present at a high rate in the endoparasitoid *C. flavipes* venom apparatus, has a disruptive effect on hemocyte behavior, suggesting that disulfide-rich venom peptides contribute to modulating the host humoral immune response during parasitism. Other putative venom toxins identified may also contribute to modulation of the host immune system.

Serpins have been previously identified in the venom of several wasp species (Danneels et al. 2010, Yan et al. 2016, Teng et al. 2016, Tang et al. 2019, Lin et al. 2019, Yokoi et al. 2017). Yan et al. (2017) demonstrated that a serpin present in the venom of the pteromalid endoparasitoid *P. puparum* binds to and inhibits serine proteases in the haemolymph of host *Pieris rapae*, resulting in inhibition of PO cascade and melanization. Thus, it is likely that venom serpins act as important weapons to protect eggs from host melanization in many parasitoid wasps, including *C. flavipes*.

Superoxide dismutase (SOD) is a metalloenzyme that defends organisms from reactive oxygen species (ROS) (Dalton et al. 1999) and which is present in venom from several parasitoids (Colinet et al. 2011, Poirié et al. 2014, Liu et al. 2017). SODs in venoms of the figitid endoparasitoid *Leptopilina boulardi* and the bethylid ectoparasitoid *Scleroderma guani* have been shown to suppress host melanization, though the precise mechanism has not been established (Colinet et al. 2011, Liu et al. 2018).

Lectins have been described to perform complex roles in parasitism by wasps. C-type lectins originating from the symbiont bracovirus of *Cotesia plutellae* and *C. rubecula* have structural similarities to host lectins and are deposited on the surface of parasitoid eggs, where they help to avoid encapsulation by host hemocytes (Glatzet al. 2003, Lee et al. 2008). C-type lectins have also been detected in the venom of the braconid wasp *Chelonus inanitus* by mass spectrometry (Vincent et al. 2010). Interestingly, parasitism of *Pieris rapae* by *Pteromalus puparum* disrupts synthesis of C-type lectins in host hemocytes and disrupts immune responses and melanization (Zhu et al. 2011, Fang et al. 2011). *C. flavipes* venom lectins may function as antagonists, competing for recognition binding sites with hemocyte lectins and disrupting their ability to detect parasitoid eggs.

Apolipophorin-III functions as a lipid transport vehicle and plays an important role as a receptor of pathogen associated molecular patterns and antimicrobial effects in the innate immune response of insects (Kim and Jin 2015, Wen et al. 2016). It has also been reported to act as a cellular immunosuppressive molecule that acts by de-adhering hemocytes (Whitten et al. 2004). Apolipophorin-III has been described in the venom glands of ichneumonid wasp *Hyposoter didymator* (Dóremus et al. 2013), and the woodwasp *Sirex noctilio* (Wang et al. 2016) as well as the venoms of assassin bugs (Heteroptera: Reduviidae) and robber flies (Diptera: Asilidae) (Walker et al. 2018a, Walker et al. 2018b, Walker et al. 2019). Presumably, *C. flavipes* venom apolipophorin-III performs role performs a role similar to the antibacterial and immune modulating properties reported for related proteins.

Prosaposin is the precursor for multiple saposin proteins that are involved in lipid recognition, especially sphingolipid degradation, and have been shown to modulate multiple aspects of the vertebrate immune system (Darmoise et al. 2010). At least one secreted saposin is associated with inducing cell death, that of the pine wood nematode *Bursaphelencus xylophilus* (Hu et al. 2019). This the first report of prosaposin in venom of a parasitoid wasp, but highly similar proteins occur in the venom of the honeybee *Apis mellifera* (Resende et al. 2013, Zhang at al. 2013). We hypothesize that *C. flavipes* venom saposins may act by inducing hemocyte cell death or by modulating other aspects of the immune system.

Chitinase have been detected in venom gland of the pteromalid wasp *Nasonia vitripennis* (Sim and Wheeler 2016). It has been suggested chitinase as a host regulation factor may perform multiple roles during the parasite life cycle, with venom chitinase upregulating the immune response against fungi and late release of chitinase by teratocytes facilitating parasitoid emergence through the host cuticle (Martinson et al. 2016, Salvador and Cônsoli 2008).

Aminopeptidases are metalloproteases that cleave N-terminal amino acids and have been widely reported in parasitoid venom (Teng et al. 2017, Becchimanzi et al. 2020, Asgari and Rivers 2011). Aminopeptidase from the venom of the ichneumonid wasp *Pimpla hypochondriaca* has been reported to have antimicrobial activity against bacteria (Dani et al. 2003), which could compensate for immunosuppression caused by other proteins and peptides in the venom cocktail.

U-BCTX-Cf23 is homologous to the '27 kDa hemolymph protein' or Gm protein-24 of the greater waxmoth *Galleria mellonella*, which is reported to be involved in immune responses to fungus (Sheehan et al. 2018) and modulation of the PO cascade (Park et al. 2005). Cf23 and another protein, Cf15, by virtue of being homologous to von Willebrand factor proteins that have been implicated in insect immune systems (Arai et al. 2013), are also hypothesized to act in immune modulation. ML domains Cf18 and Cf25 Transferrin (Yoshiga et al. 1997).

4.2. Nutritional interference by venom proteins

We found several putative venom proteins that are likely to have a role in digestion or catabolism. The role of such proteins in parasitism remains elusive, but likely involves directly mobilizing nutrients from host tissues to facilitate growth of parasitoid larvae, or indirect effects on the immune system (Heavner et al. 2013).

Cathepsin L is an enzyme usually often located in lysosomal compartments, but which is also released into the extracellular space by apoptosing specialized hemocytes called macrogranulocytes (Zhai and Zhao 2012). It is known to have key roles in fat body degradation and tissue remodeling in unparasitized insects, and also fat body degradation in parasitized insects. For example, Becchimanzi et al. (2017) found increased cathepsin L activity on the surface of fat bodies and associated with a layer of host hemocytes, as

well as increased expression of cathepsin L in the haemolymph of parasitized larvae. Although these data were interpreted to reflect increased expression of host cathepsin L due to larval reprogramming of host physiology, our data suggest that cathepsin L present injected as a venom component may also contribute to host fat body degradation.

Serine carboxypeptidase-like enzymes have been previously identified in wasp and bees' venoms (Laurino et al. 2016, Teng et al. 2016, Danneels et al. 2015; van Vaerenbergh et al., 2015). In *Sitodiplosis mosellana* (Diptera: Cecidomyiidae), serine carboxypeptidases are evolved in extracellular digestion of proteins prior ingestion or degradation of vitellogenin (Mittapalli et al. 2006). *C. flavipes* venom serine carboxypeptidase may act to mobilize amino acids to foster parasitoid growth.

The enzyme trehalase hydrolyzes the disaccharide trehalose, the main storage sugar of insects, into the monosaccharide glucose (Shukla et al. 2015). Trehalase has been detected in the venom of various parasitoid species (Asgari and Rivers 2011, Zhao et al. 2017, Poirié et al. 2014, Sim and Wheeler 2016) where it is proposed to have a digestive function, providing glucose for the developing wasp larvae (Parkinson et al. 2003, Danneels et al. 2010).

4.3. Neurotoxins

Although the venoms of koinobiont wasps such as *C. flavipes* do not generally possess the strong paralytic toxins that are typical of idiobiont species, they nevertheless often cause short-term paralysis of the host that facilitates oviposition (de Souza et al. 2019, Quicke and Butcher 2021). We found that *C. flavipes* venom contains multiple disulfide-rich peptides similar to venom neurotoxins of other arthropod toxins, including peptides with primary structures similar to ICKs and von Willebrand factor domain peptides. We hypothesize these function in neuronal modulation, either contributing to short-term host paralysis or more subtle modulation of host behavior. However, these putative toxins may have different functions, as evidenced by our finding that Cf4 modulates the host humoral defense system. Further studies are required to determine the function of other *C. flavipes* venom peptides.

4.4. Use of endoparasitoid venom toxins in biotechnology

Due to the wide diversity of parasitic factors that act during the host regulation process, parasitoids are considered a large reservoir of biomolecules with biotechnological potential that can be considered and investigated for pest control (Di Lelio et al. 2014). For example, a gene originating from the symbiotic bracovirus of *Cotesia vestalis* (Hymenoptera: Braconidae) was used to generate a transgenic tobacco plant with the expression of the cystatin CpBV-CST1 (Kim et al. 2016). Among other changes, it was observed that, after feeding on the transformed plant, the host larvae *Spodoptera exigua* (Lepidoptera: Noctuidae) showed high mortality in their first instars. This transformed plant has also displayed toxicity to other pest insects such as *Helicoverpa assulta* (Lepidoptera: Noctuidae) and *Myzus persicae* (Hemiptera: Aphididae) (Kim et al. 2016). Another gene from the polyDNAvirus of *Cotesia rubecula* (CrV1) was used to transform a species of baculovirus (AcMNPV-CrV1). The recombinant entomopathogen displayed intensified insecticidal power intensified over *Pieris rapae* (Linnaeus, 1758) (Lepidoptera: Pieridae) (WEI et al. 2016a, WEI et al. 2016b). Teratocyte chitinases have also been developed as potential pesticides (Rossi et al. 2012). A recent work showed that transgenic tobacco plants transformed with a chitlectin from teratocyte have increased resistance to a wide range of agricultural pests (Merlin et al. 2020). In contrast, biotechnological applications based on the venom toxins of endoparasitoids are scarce.

We investigated if Cf4 could have an insecticidal potential when ingested by *D. saccharalis* neonates. We found that insect viability and leaf consumption were lower when the insects ingested the peptide. Possibly, Cf4 exerts toxic effects in the gut, or is translocated to the *D. saccharalis* hemolymph. If so, Cf4 may have utility similar to established toxins used in pest control, like the *Bacillus thuringiensis*-derived toxins Cry and VIP. Such toxins are naturally produced by the bacteria species *Bacillus thuringiensis* and, besides being highly specific, present a high mortality by ingestion either when produced in transgenic plants or if applied by spraying (Chakroun et al. 2016, Jurat-Fuentes and Crickmore 2017). However, further studies are required to test if Cf4 exerts toxic effects or alternately induces increased mortality because *D. saccharalis* avoids eating leaves coated in Cf4.

Endoparasitoid venom toxins have distinct biological functions with a single unifying objective: the establishment of parasitoid offspring inside the host. This study details the primary structures of putative venom toxins of the economically important endoparasitoid wasp *C. flavipes* and describes the characterization of one of these, the disulfide-rich venom peptide Cf4. The mechanism of action of Cf4 remains unclear, but together to the other molecules present in the venom cocktail, it prevents the parasitoid egg encapsulation by the host immune system. Further, our results strongly suggest that Cf4 is active against cellular immune response and when ingested by neonate *D. saccharalis* results in reduced feeding and increased mortality.

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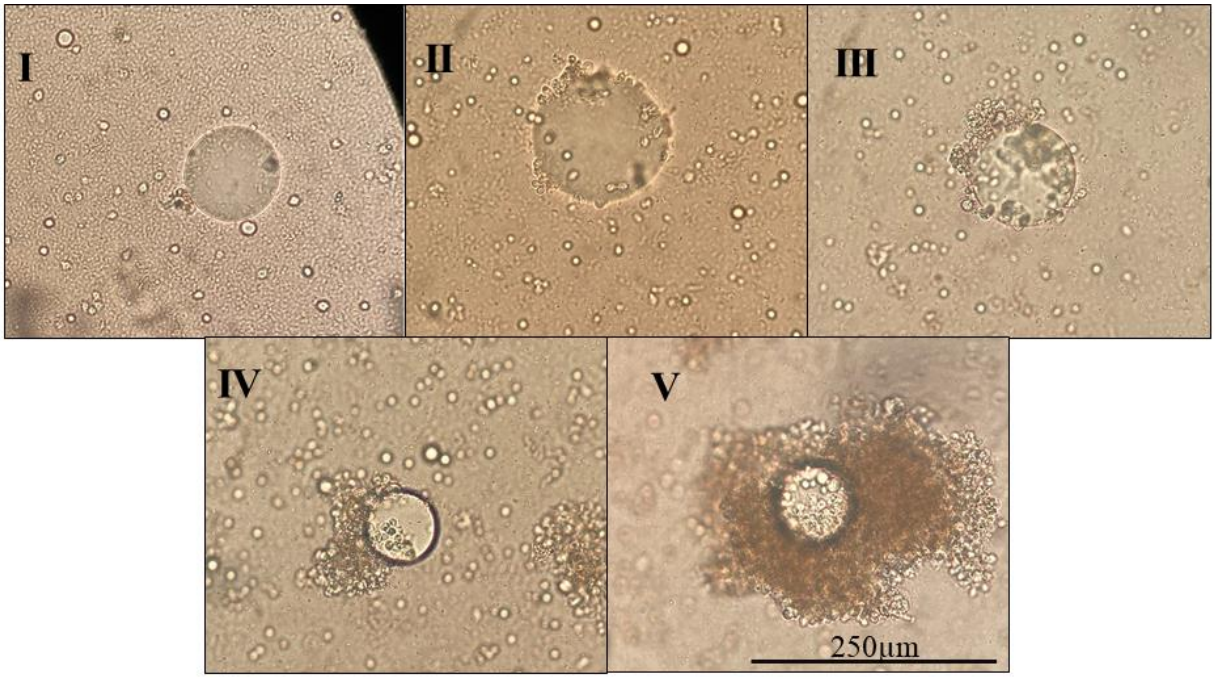
Supplementary data



Suppl. Fig 2. Encapsulation classed. class **I** are the beads with at most ten hemocytes attached; class **II** are the beads with 10-50 hemocytes attached; class **III** are the beads with more than 50 hemocytes attached and covered with layer of mostly three cells; class **IV** are beads covered with more than 3 layers of cells thinner than the bead diameter; class **V** are beads covered a layer s cells larger than he bead diameter.

Chapter V - Understanding teratocytes-derived peptides from *Cotesia flavipes* impairing *Diatraea saccharalis* cellular immune response and hints for pesticide discovery

Abstract

Some koinobiont endoparasitoids wasps are known for releasing teratocytes inside their hosts. Teratocytes are special cells released from the serosa, an extra-embrionary layer of “sister cells” that surround the embryos during embryonic development. Teratocytes are known for releasing a cocktail of molecules at act in host regulation. In this study, we selected five peptides previously identified in the teratocytes of *Cotesia flavipes* Cameron 1891 (Hymenoptera: Braconidae), produced them using heterologous expression or solid phase synthesis, and analyzed their involvement in host regulation as well their potential as insecticides after ingested by *Diatraea saccharalis* (Fabricius 1794) (Lepidoptera: Crambidae) larvae. Using a consistent proteomic approach, the quality of the produced peptides was verified in terms of the formation of the predicted disulfide bridges. Biological assays identified the function of ICK-like peptides as cellular immune suppressor factors by disrupting hemocyte nodulation rate. The peptides tested did not influence melanisation of host hemolymph. Results also showed neonate *D. saccharalis* experienced higher mortality rates after ingesting ICK-like peptides. Taken together, these findings indicate that *C. flavipes* teratocytes secrete products have special function on disrupting nodulation by hemocytes. Complementarily, we discuss the potential of such molecules for pesticide prospection.

Keywords: Inhibitor cysteine knot, host regulation, nodulation, phenol oxidase

1. Introduction

Parasitoids wasps are hymenopteran insects whose immature stage develops using usually only one host as food source to complete their cycle. The applied biological control with parasitoid wasps, *i.e.* the production of parasitoids in controlled conditions with consequent release in agroecosystems, is a viable and ecofriendly option that can replace other methods for pest control. Larval parasitoids are widely used as biological control agents (BCA) in crop protection (Barratt et al. 2018, van Lenteren et al. 2018). For instance, *Cotesia* spp. are among the most used BCA in applied biological programs, especially in South America and China (van Lenteren et al. 2018).

Parasitoids can be classified according biological characteristics of the parasitic stage. Ectoparasitoids develop outside their hosts while endoparasitoids develop inside their hosts. Solitary parasitoids develop with only one larva per host, while gregarious parasitoids develop in groups in the same host. Idiobionts immobilize their hosts during parasitic stage, while koinobionts allow the host to continue feeding and moving during parasitic stage. Each parasitism strategy requires specific modes of host regulation. For example, ectoparasitoids do not use teratocytes as a factor for host regulation, and idiobionts immediately immobilize their hosts by the injection of stronger and deadly venoms in relation to the venom of koinobionts (Pennacchio and Strand 2006, Becchimanzi et al. 2020).

The factors involved in host regulation can be classified according their origin into maternal or embryonic (Strand 2014, Ali et al. 2015). Briefly, maternal mechanisms consist on the injection of venom, calix fluids, and symbiotic viruses by the female parasitoid into the host during oviposition (Asgari and Rivers 2011, Gundersen-Rindal et al. 2013, Moreau and Asgari 2015, Zhu et al. 2018). The host regulation mechanisms of embryonic origin are related to secretions of the developing parasitoid larvae and the regulatory activity of teratocytes (Dahlman 1990, Herzner et al. 2013, Strand 2014).

Teratocytes are extraembryonic sister cells dissociated from the serosa, a layer of cells that surrounds the embryos, after parasitoid larvae hatching inside the host (Dahlman 1990, Strand 2014, Glupov and Kryukova 2016). Among teratocyte functions, we highlight immunosuppression (Ali et al. 2015, Kato et al. 2016), control of the endocrine system (Merlin and Cônsoli 2019), overall regulation of nutrition (Cônsoli et al.

2001), control of protein synthesis (Kadono-Okuda 1998), metamorphosis abnormalities (Shi et al. 2015), enzymatic degradation of fatty body (Nakamatsu et al. 2002) and others (Rana et al. 2002, Caccia et al. 2012, Shi et al. 2016).

One of main functions of teratocytes is the disruption of host immune system (Strand 2014). For example, the parasitoid *Cotesia vestalis* (Haliday 1834) (Hymenoptera: Braconidae) knocks out the phenoloxidase (PO) activation pathway by releasing a trypsin inhibitor-like protein (*CvT-TIL*), an inhibitor of proteases that activates the inactive forms of PO (Gu et al. 2019). Beside proteins, teratocytes also express microRNAs (mRNA) that disrupts the expression of ecdysone receptors, a key type of receptor for insect molting and PO activity (Genta et al. 2010, Wang et al. 2018).

Furthermore, teratocytes from *C. vesta*lis also express immune defensive peptides like rTSVP-8, a variety of defensins, CvT-PGRP1 and CvT-PGRP2 that inhibit bacterial growth (Gao et al. 2016). These observations point out the relevance of teratocytes as a source of useful molecules for biotechnological purposes. Applying a robust proteo-transcriptomic approach, we found a variety of molecules from teratocytes from *Cotesia flavipes* Cameron 1891 (Hymenoptera: Braconidae), including immune suppressive, polydnaviruses-associate peptides and serine protease inhibitors (serpins) (unpublished data).

Even still not so deeply explored, there are some cases of agricultural biotechnological exploitation of teratocytes-derived molecules. The first insecticidal evaluation of a teratocyte peptide was performed by Maiti et al. (2003), where the authors expressed a teratocyte secretory protein (TSP14) in transgenic tobacco plants, inducing resistance against *Heliothis virescens* (Fabricius, 1777) (Lepidoptera: Noctuidae) and *Manduca sexta* (Linnaeus, 1763) (Lepidoptera: Sphingidae). In an earlier study of our group, it was verified the insecticidal effect of chitolectin (*Tn*Chit) from the teratocytes of *Toxoneuron nigriceps* (Viereck) (Hymenoptera: Braconidae) inserted into tobacco plants (Rossi et al. 2012). Recently, the insecticide potential of *Tn*Chit was also verified in other non-permissive hosts considered as agricultural pests feeding on transgenic solanum plants expressing *Tn*Chit (Merlin et al. 2020).

Based on our previous proteotranscriptomic study (unpublished data), we selected some of the most expressed transcripts that code for peptides in the teratocytes of *C.*

flavipes for a further functional and biotechnological investigation. In this study, we expressed a *C. flavipes* teratocyte Inhibitor cystine knot variant (CftICKv) and a von Willebrand Factor (CftWF) using periplasmic expression system in *E. coli* and synthetized an inhibitor cystine knot (CftICK), an unknown peptide (CftUP) and an uncharacterized peptide (CftUncP) using a solid phase synthesizer. We investigated the immune suppressive properties of these selected peptides at humoral (PO activity) and cellular (hemocytes number and activity) levels. In addition, aiming to verify the insecticidal potential of the selected peptides, we spread the peptides on the surfaces of sugarcane leaves and fed neonates *Diatraea saccharalis* (Fabricius 1794), the sugarcane borer, an important sugarcane pest and a permissive host of *C. flavipes*.

2. Material and Methods

2.1. Peptide synthesis and recombinant expression

We selected the most abundant peptides revealed by a proteotranscriptomic investigation of the teratocytes of *C. flavipes* and the hemolymph of parasitized *D. saccharalis* larvae and according their mature size, we synthetized those peptides using phase solid synthesis (shorter than 45 residues) or recombinant periplasmatic expression. The putative inhibitor cystine knot (CftICK), an unknown peptide (CftUP) and an uncharacterized peptide (CftUncP) were synthetized by solid phase synthesis. The inhibitor cysteine knot variant (CftICKv) and a von Willebrand Factor (CftWF) were produced by recombinant periplasmatic expression. Note that CftUP and CftUncP may be also considered as ICKs according their molecular features (See supplementay material).

For solid phase chemical synthesis, peptides shorter than 35 residues ought to be preferentially selected (Upert et al. 2014), but we successfully synthetized CftUncP with 42 residues. The crude synthetic product was cleaved from the resin, desalted, and subjected to an oxidation process (Reynaud et al. 2020). After desalting, high-performance liquid chromatography (HPLC) and Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-TOF) matrix-assisted laser desorption/ionization mass analysis were performed to validate the

presence of the expected mass and for selection of the correct fractions (Supplementary Figure 3, 4 and 5).

For recombinant expression, we used optimized protocols described by Klint et al. (2013) and Saez et al. (2017). Genes encoding the peptides were codon optimized, synthesized and subcloned into the pLIC-MBP plasmids (Invitrogen, Thermo Fisher Scientific, USA) by GeneArt (Thermo Fischer Scientific). For adequate periplasmatic expression, plasmids were designed with a fusion protein containing a MalE signal sequence (Cabrita et al. 2006, Saez et al. 2017). To optimize affinity purification and promote peptide solubility, an N-terminal His6 tag and a maltose binding protein (MBP) were included, respectively. Finally, the fusion protein contained a Tobacco Etch Virus protease (TEV) recognition site that allowed the cleavage between the teratocyte peptides and the His6-MBP fused fragment. To improve TEV protease cleavage of the fusion protein, one extra glycine (G) residue was inserted at the N-terminus of CftWF.

For protein expression, the plasmids were reconstituted in a final concentration of 100 ng μL^{-1} and were individually inserted into *E. coli* BL21 (DE3) competent cells by heat shock followed by ampicillin (100 µg mL^{-1}) selection LB agar plates. One transformed colony of each construct was grown overnight in 5 mL LB amended with 100 µg mL^{-1} ampicillin and 0.01% glycerol in a 50 mL Falcon tube at 37°C with shaking (250 rpm). After overnight culture (~16 hours), we performed a pilot expression of an autoinduction protocol started by inoculating 0.25 mL of the overnight grown cultures in 5 ml L of ZYP-5052 medium containing 100 µg mL^{-1} ampicillin (Jin et al. 2020). Expression culture (5 mL) was inoculated with 0.25 mL of the overnight culture and incubated at 24°C with shaking (250 rpm) for approximately 3 h (culture OD_{600} = 0.8-1.0). Next, cell cultures were incubated overnight at 16°C overnight (250 rpm) and protein expression was verified after ~16 h.

For observation of protein expression, cells were harvested by centrifugation (6,000 rpm, 5 min, room temperature) of 1 mL of bacterial cultures and the supernatants were discarded. Pellets were resuspended in a lysozyme solution (2 mg/mL) and incubated at 37°C for 30 min to lyse cells. Finally, the lysate was centrifuged, and the supernatants were collected and loaded into 10% SDS-PAGE (Sambrook and Russel 2001).

Gels were overnight stained with Coomassie reagent (40% MeOH, 10% acetic acid, 0.1% w/v Coomassie Brilliant Blue R250) gently shook in an orbital shaker and distained in an aqueous solution containing 40% MeOH and 10% acetic acid with shaking. After confirming the expression of peptides in the soluble form, we started a large-scale expression under the same conditions.

For large-scale expression, each bacterial construct were grown overnight in 50 mL of LB amended with 10 µg mL^{-1} ampicillin and 0.01% glycerol with shaking (180 rpm) at 37°C. Cell cultures were used to inoculate 1 L of autoinduction expression culture cultivated in the same conditions of the pilot expression assay. Following expression, cells were harvested by centrifugation at 6,000 g and the supernatants were discarded. Next, pellets were dissolved at the proportion of 1 g of cell pellet in 20 mL of TN buffer (0.4 M NaCl, 40 mM Tris-HCl, pH 7, 15 mM imidazole, pH 7.5). For collecting only the soluble fraction, cells were lysed in a cell disrupter (35 kpsi, 4°C), the flow though was collected and centrifuged (18,500 rpm, 15 min, 4°C), the pellet was discarded, and supernatants were transferred to new vials for Ni-NTA affinity chromatography.

2.2. Ni-NTA affinity chromatography

Cell lysates prepared in the previous step contain the soluble form of recombinant peptides and bacterial contaminants. To purify the recombinant peptides, we performed an affinity chromatography using a Ni-nitrilotriacetic acid (NTA) column since the recombinant peptides were fusioned to a polyhistidine tag at the N-terminus that binds to Ni. In this type of chromatography, weakly bound contaminants are washed from the column with the washing buffer with low concentration of imidazole (TN buffer pH 7.5, with 15 mM imidazole), while the fusion peptides remain bound to the column. Following, fusion peptides are eluted from the column with the elution buffer containing high concentration of imidazole (TN buffer pH 7.5 with 500 mM Imidazole), which disrupts the bound between polyhistidine tag and Ni^{2+}.

Briefly, Ni-NTA beads were added to a column (200 mL) and washed 3x with 15 mL of water. Following, 20 mL 100 mM $NiSO_4$ were loaded into the column to cover the resin and the flux were interrupted for 10 minutes. This Ni recharge of the resin was repeated twice and, after Ni recharge, column was washed with 20 mL of H_2O twice. Next,

we equilibrated the column with a new wash with 20 mL of TN buffer and loaded the column with the cell lysates that were mixed and incubated for 5 min for fusion protein to bind to Ni-beads. Fusion proteins bound to Ni-beads were eluted twice with 10 mL of the TN buffer altered to 500 mM imidazole.

The eluates collected were concentrated, and imidazole removed from the samples using an Amicon centrifugal ultra filter (30 kDa) (Amicon®) and TN buffer without imidazole. In order to cleave the desired peptide from fusion protein, TEV protease (1 mg mL^{-1}) prepared in redox buffer [0.6 mM reduced glutathione (GSH) and 0.4 mM oxidized glutathione (GSSG) in TN buffer, pH 8.0] was added at the final concentration of 25 µg mL^{-1}. After cleavage (~16 h, room temperature), peptides were purified using HPLC on a Shimadzu HPLC system (Kyoto, Japan) controlled by Shimadzu LC solution software. Samples were purified on a C4 Jupiter column (10 µm, 300 Å, 250×10 mm, #00G- 4055-N0, Phenomenex, Torrance, California, USA) using a gradient of 5%–55% solvent B [0.043% trifluoroacetic acid (TFA) in 90% acetonitrile (ACN)] in solvent A (0.05% TFA) over 40 min (3 mL min^{-1}). Finally, masses confirmed by Electro Spray Ionization mass spectrometer (ESI-MS) (Supplementary Fig. 1 and 2) and peptide concentration estimated from A280 measured on a Nanodrop spectrophotometer.

2.3. Phenol oxidase activity

For *in vivo* assay of PO, 10 µL (containing 10 µg) of each peptide were individually injected in 5th instar larvae. After 24 h, hemolymphs from injected larvae were centrifuged (1,000g, 5 min, 4ºC) and the supernatants were collected and cell pellet was discarded. Subsequently, 2 µL of cell free hemolymph were transferred to a 96-well plate and mixed to 198 µL of PO substrate (5 mM L-Dopa, 10 mM sodium cacodylate, 5 mM $CaCl_2$ pH 7.0). Plates were incubated at 30°C in a Multiskan Sky Microplate Spectrophotometer (Thermofisher) coupled to the software SkanIt v.6.1 for absorbance reading. PO activity was determined by continuously measuring the absorbance at 490nm in 30 seg intervals for 60 min. Only the linear phase of absorbance reading (Absorbance vs time; $R^2 \geq 0.99$) was used for calculating PO activity. One unit of PO (U) was determined as the amount of enzyme necessary to increase 0.001 units of absorbance at 490 nm per minute. Each

assay was performed in a complete randomized design with ten replicates composed by three larvae each.

2.4. Hemocytes behavior assessment

To assess *in vitro* hemocyte encapsulation capability facing CftICK, CftUP, CftUncP, CftICKv or CftWF, 20 µL of hemolymph from 5th instar *D. saccharalis* larvae was collected and mixed with 40 µL InsectXpress™ (Lonza®) culture medium inside a PCR tube (200 µL). Next, 20 µL of 0.1% w/v A-25 sephadex beads and the peptides were individually added and homogenized. To keep beads in contact to the hemocytes, we mixed the samples for 2 h at room temperature and 100 rpm. We assigned to five encapsulation classes (I–V) according to the thickness of the capsule were observed and recorded under the phase contrast microscope (Zhang et al. 2005, Wu et al. 2008, Teng et al. 2016, Li et al. 2018). Class **I** are the beads with at most ten hemocytes attached; class **II** are the beads with 10-50 hemocytes attached; class **III** are the beads with more than 50 hemocytes attached and covered with at most a layer of three cells; class **IV** are beads covered with more than 3 layers of cells thinner than the bead diameter; class **V** are beads covered a layer of cells larger than he bead diameter. The encapsulation index (EI) was calculated by the formula EI (%)= [(Σ defined classes/ Σ number of defined beads) *100]/5.

To assess hemocyte adhesion, 10 µL of hemolymph extracted from *D. saccharalis* larvae after 24 h injection of 10, 1 and 0.1 µg of CftICKv were placed in glass slides and incubated at room temperature for 30 min. For removing debris and unadhered cell, the hemolymph was washed with PBS, cells were fixed with 5% methanol in anticoagulant solution for 5 min (Manachini et al. 2011) and stained with Giemsia for 10 min. Finally, glass slide was washed with anticoagulant buffer and covered with a clean coverslip. For obtaining the images, slides were photographed under phase contrast microscopy (Zeiss® Axio Imager A2) and the data was considered as the percent of total area covered by adhered cells on the glass slides measured by the software ImageJ.

Finally, total hemocyte count (THC) of *D. saccharalis* larvae injected with 10 µg of peptides was performed using a Neubauer Chamber (HBG® 9020-01) by counting four

quadrants each sample and estimating the total cell count (TTC) using the formula: cells μL^{-1}=[(Σcells×dilution×10,000)/Σquadrants]/1000.

2.5. Biotechnological investigation of C. flavipes teratocytes peptides

Sugarcane leaf disc of 3.14 cm^2 area of the IAC95-5000 variety were covered with ~40 µL of 100 µg mL^{-1} of peptides and as control we used deionized water. Leaves were superficially air dried for 10 min and subsequently placed in 24-well plates containing 1 mL of agar 1% at the bottom. After three days, we verified the leaf consumption and larval viability by touching larvae with a soft brush. Non-responsive larvae were considered as unviable. Leaf consumption was measured as the damaged leaf area, using ImageJ.

2.6. Data analysis

All experiments were conducted in a completely randomized design. Data were submitted to the Bartlett tests for homoscedasticity and Kolmogorov tests for normality before analysis of variance (ANOVA). After checking the requirements for ANOVA, means were compared by Dunn's test for non-parametric data or Dunnet's test for parametric data. Statistical analysis were performed and plotted using GraphPadPrism 9.

3. Results

3.1. Peptides production

We previously identified 5 peptides produced by *C. flavipes* teratocytes, a complex source of peptides since the peptides produced by this cell-type are released into host hemolymph, an environment that contains a cocktail of host peptides with life-keeping functions. Host regulation factors are not necessarily toxic, but most of them have highly specific functions to manipulate host physiology even in almost undetectable amounts. Aiming to understand the structural and functional features of peptides produced by teratocytes of *C. flavipes*, we selected five peptides of the most abundant peptides for heterologous expression or synthesis.

Two teratocyte peptides, CftICKv and CftWF, were produced by overexpression of His6-MBP-fusioned peptides using *E. coli* periplasmatic expression, purified using nickel affinity chromatography, released from fusioned tags (His6 and MBP) using TEV protease

and finally purified by RP-HPLC (Fig. 1A, B and C for CftICKv and Fig.1 D, E and F for CftWF). For both heterologous peptides, the expected oxidized mass perfectly matched by ESI-MS (Supp. Fig. 1 and 2)**.**

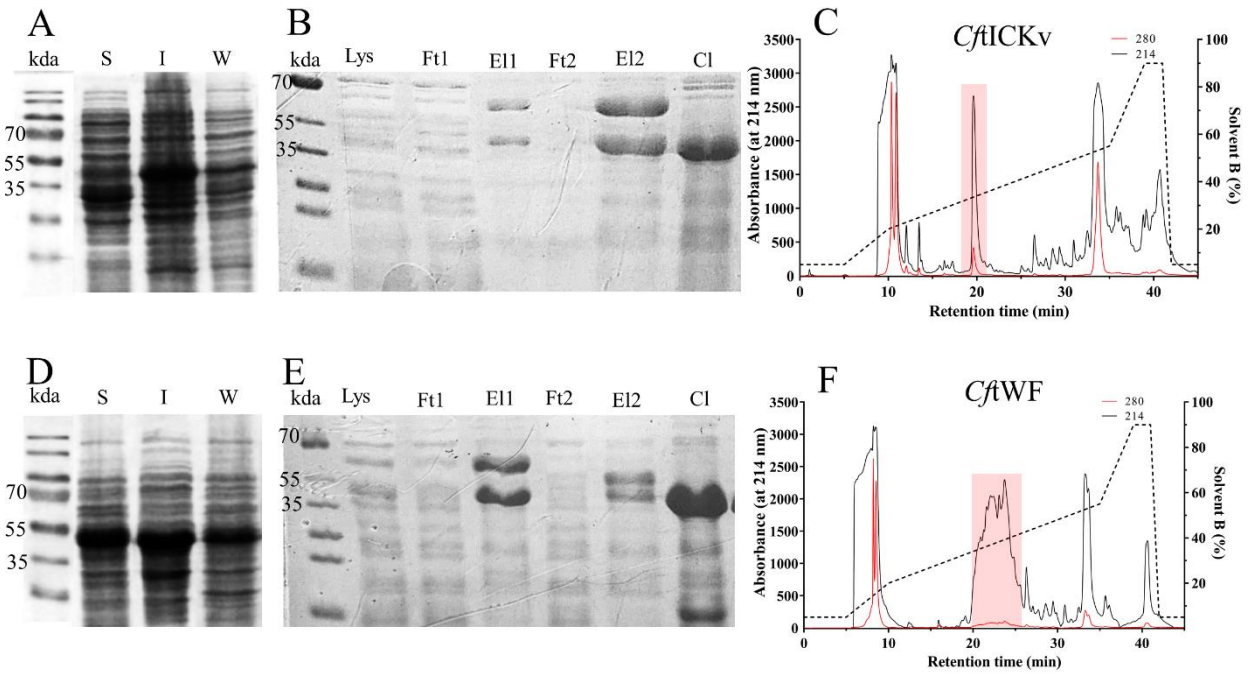


Figure 1. Heterologous expression of the teratocyte derived peptides *Cft*ICKv (A to C) and *Cft*WF (D to F). A and **D-** Expression test of soluble (S), insoluble (I) and whole cell (W) of transformed *E. coli*. The expected band, considering fusion protein plus peptide, has a size near to 50 kDa. **B** and **E-** Purification processes in NI-NTA column. Original lysate (Lys), Flow through 1 (Ft1), Eluent 1 (El1), Flow through 2 (Ft2), Eluent 2 (El2), Cleavage (Cl). **C** and **F-** HPLC of the soluble fractions passed through NI-NTA column and with fusion protein lysate with TEV.

For the synthetic peptides we performed two batches of purification by RP-HPLC to select the fractions with the correct reduced mass, all confirmed by MALDI-TOF-TOF (Supplementary Fig 3, 4 and 5). After the oxidation process, the folded mass of CftUP was confirmed by an addition run by LC-MS-MS of the purified peptide ($M+4H^+$) (Supplementary Fig. 3). The oxidized mass of CftICK had difficulties to be confirmed by LC-MS-MS, but the oxidized mass is acorrect according ESI-MS assay ($M+3H^+$ to $M+6H^+$) (Supplementary Fig. 4). Finally, CftUncP could be compared to its native form and the folded mass confirmed by MALDI-TOF-TOF ($M+4H^+$ and $M+5H^+$) (Supplementary Fig. 5).

3.2. Cellular immune system

The encapsulation activity of hemocytes was assessed by an *in vitro* approach since injection and recovery of beads may be difficult and imprecise (Li et al. 2018). Thus, the approach we used in this paper was optimized to generate the most reliable encapsulation answer as possible. We classified the encapsulated beads into five indexes, based on the amounts of hemocytes and thickness of hemocyte layer over the surface of the beads. Comparing to the control with PBS, we observed that the peptides CftUP, CftUncP and CftICK reduced the encapsulation index at 40.4%, 43.0% and 60.0% when the assays were conducted with 10 µg of each peptide ($\chi^2 = 77.87$; df= 15; $p < 0.001$) (Fig. 2).

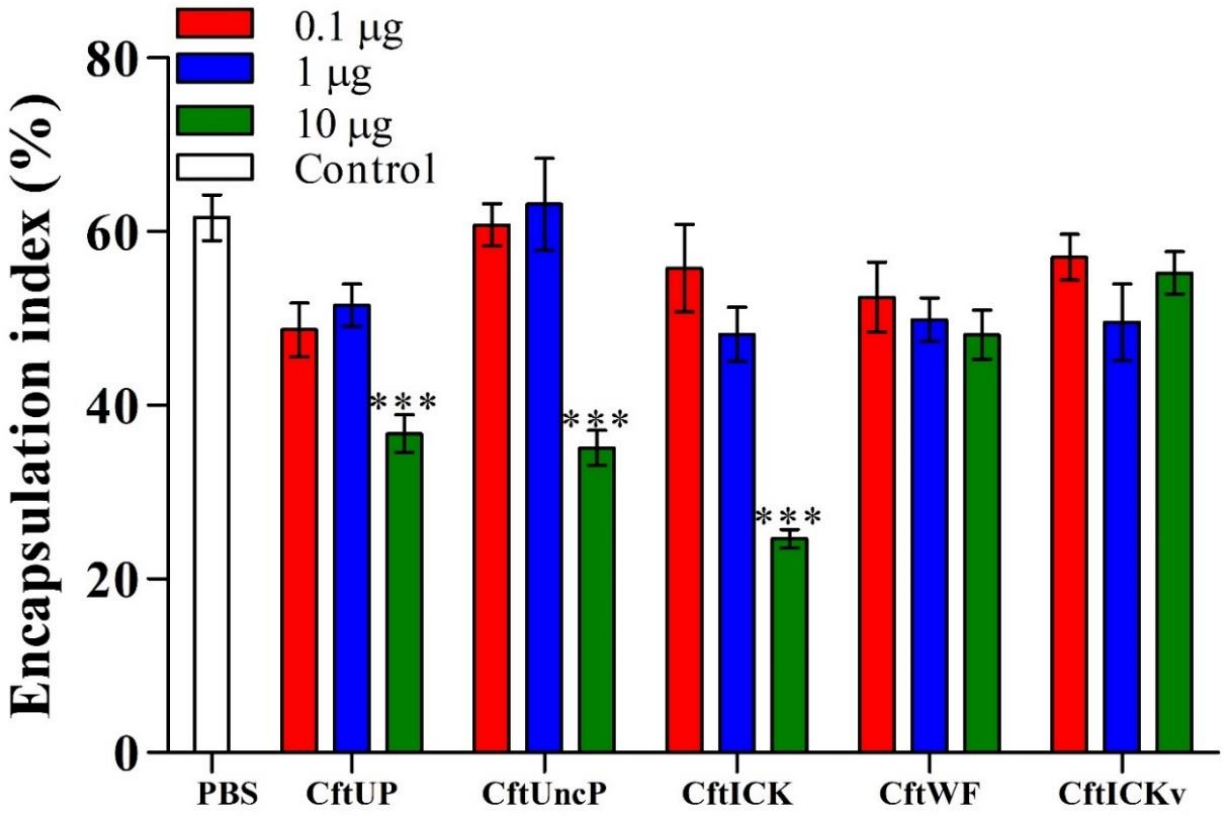


Figure 2. Influence of *Cotesia flavipes* teratocytes derived peptides on *Diatraea saccharalis* hemocyte *in vitro* encapsulation index (%). Bars represent means ± standard errors. Asterisks, if present, above the bars indicate significant differences compared to control treatment with PBS (*p< 0.05, **p< 0.01, ***p<0.001, Dunn's multiple comparison test).

To investigate the influence of the selected teratocyte-derived peptides over total hemocyte counting (THC), 10 µg of each peptide was injected into one larval *D. saccharalis* and THC was evaluated after 24 h. None of the peptides influenced THC ($F_{5,54}$= 1.760, p= 0.1368) (Fig. 3). In addition, a dose dependent injection (10, 1 and 0.1 µg)) of CftICKv did not interfere to the hemocyte adhesion capability ($F_{3,32}$=1.437, p=0.2503) (Supplementary Fig. 6).

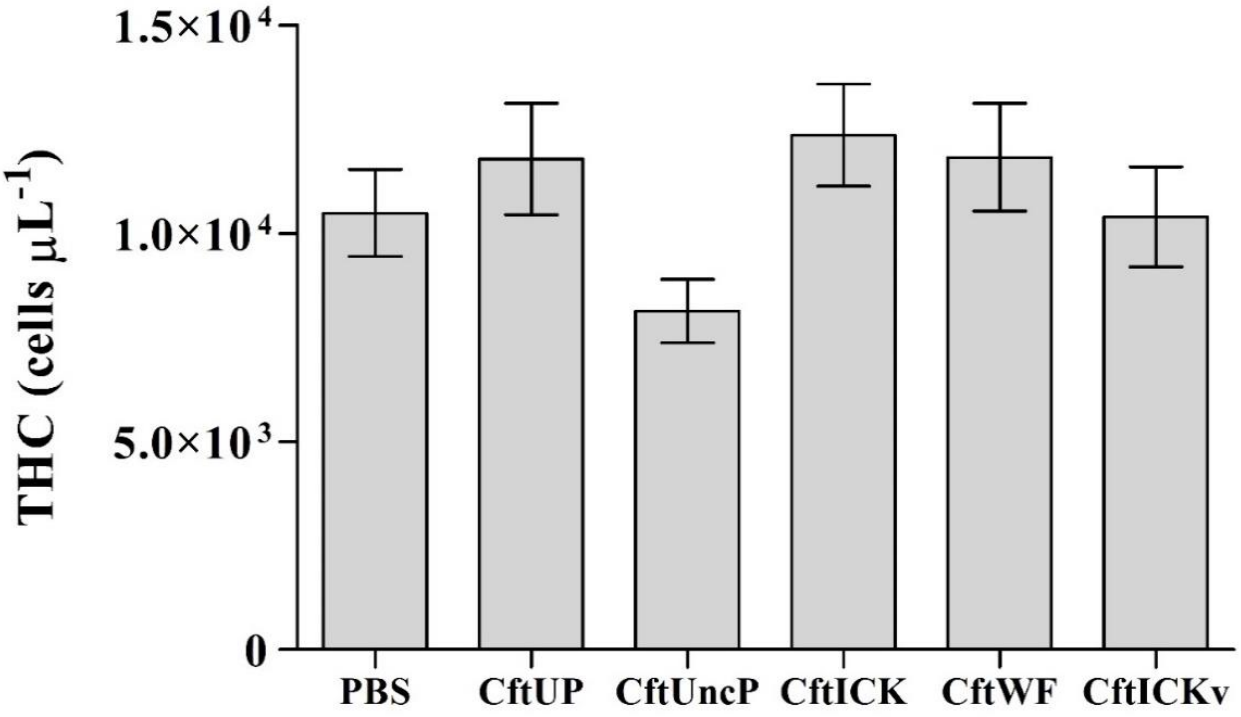


Figure 3. Total hemocyte count (THC) of *D. saccharalis* hemocytes inject with 10 µg of the teratocyte peptides. Bars represent means ± standard errors. Asterisks above the bars, if present, indicate significant differences compared to the control (PBS) (*p < 0.05, **p < 0.01, ***p<0.001, Dunnett's multiple comparison test).

3.3. Humoral immune system

In order to verify the influence of the selected teratocyte-derived peptides over PO activity, we injected 10 µg of each peptide into 5th instar *D. saccharalis* and measured PO activity after 24 h. PO activity of the larvae did not change facing any of the peptides ($F_{5,83}$=2.157, p= 0.066) (Fig. 4).

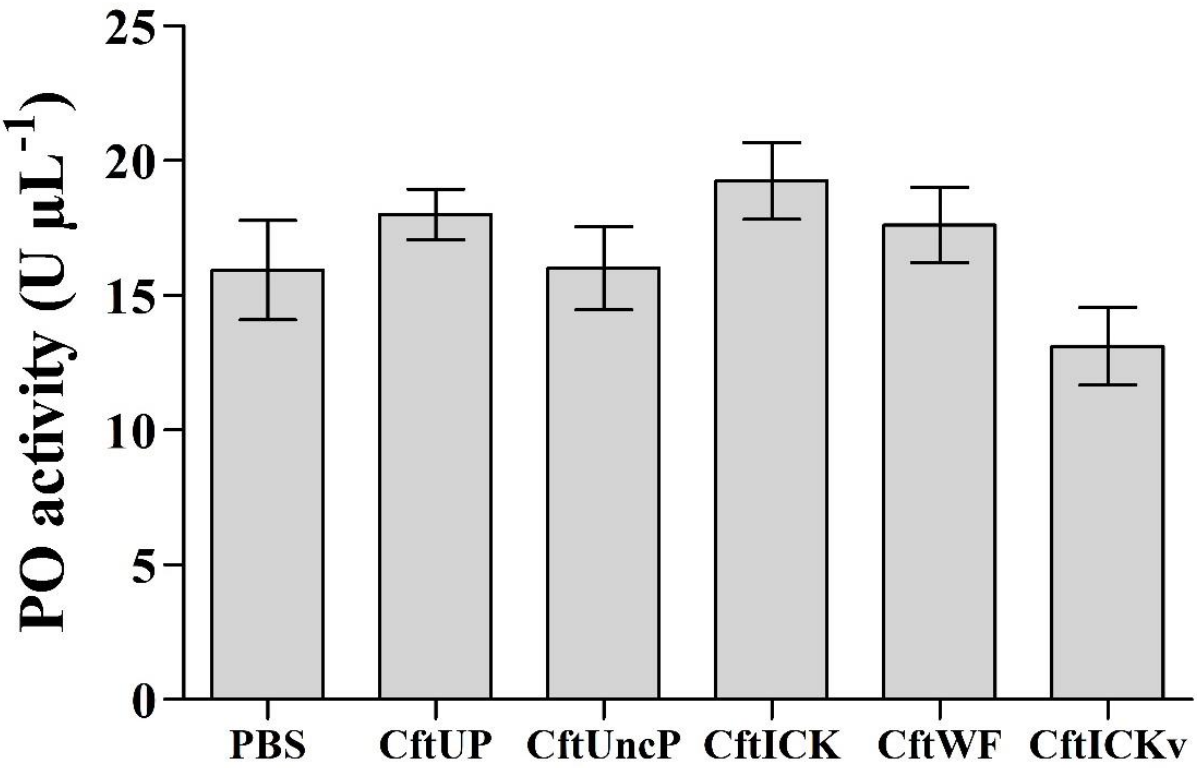


Figure 4. Phenol oxidase (PO) activity of *D. saccharalis* injected with 10 µg of *Cft*ICK, *Cft*UP, *Cft*UncP, *Cft*ICKv or *Cft*WF with reads performed 24 h after injection. Bars represent means ± standard errors. Asterisks, if present, above the bars indicate significant differences compared to the control (PBS) (*p<0.05, **p<0.01, ***p<0.001, Dunnet's multiple comparison test).

3.4. Insecticidal activity

To achieve insecticidal potential of the molecules, sugarcane leaves covered with 100 µg mL^{-1} were provided as food to *D. saccharalis* neonates. The leaf consumption and insecticidal activity were assessed three days later. We found that neonates avoided to ingest the peptides ($F_{5,30}$=101.1, p<0.0001) (Fig. 5A) and even with a low consumption, CftUP, CftUncP and CftICK presented a higher degree of mortality when compared to the control treatment with PBS ($F_{5,30}$= 15.12, p<0.001) (Fig. 5B), pointing that a small amount of thee peptides was enough to be harmful to the neonate *D. saccharalis*.

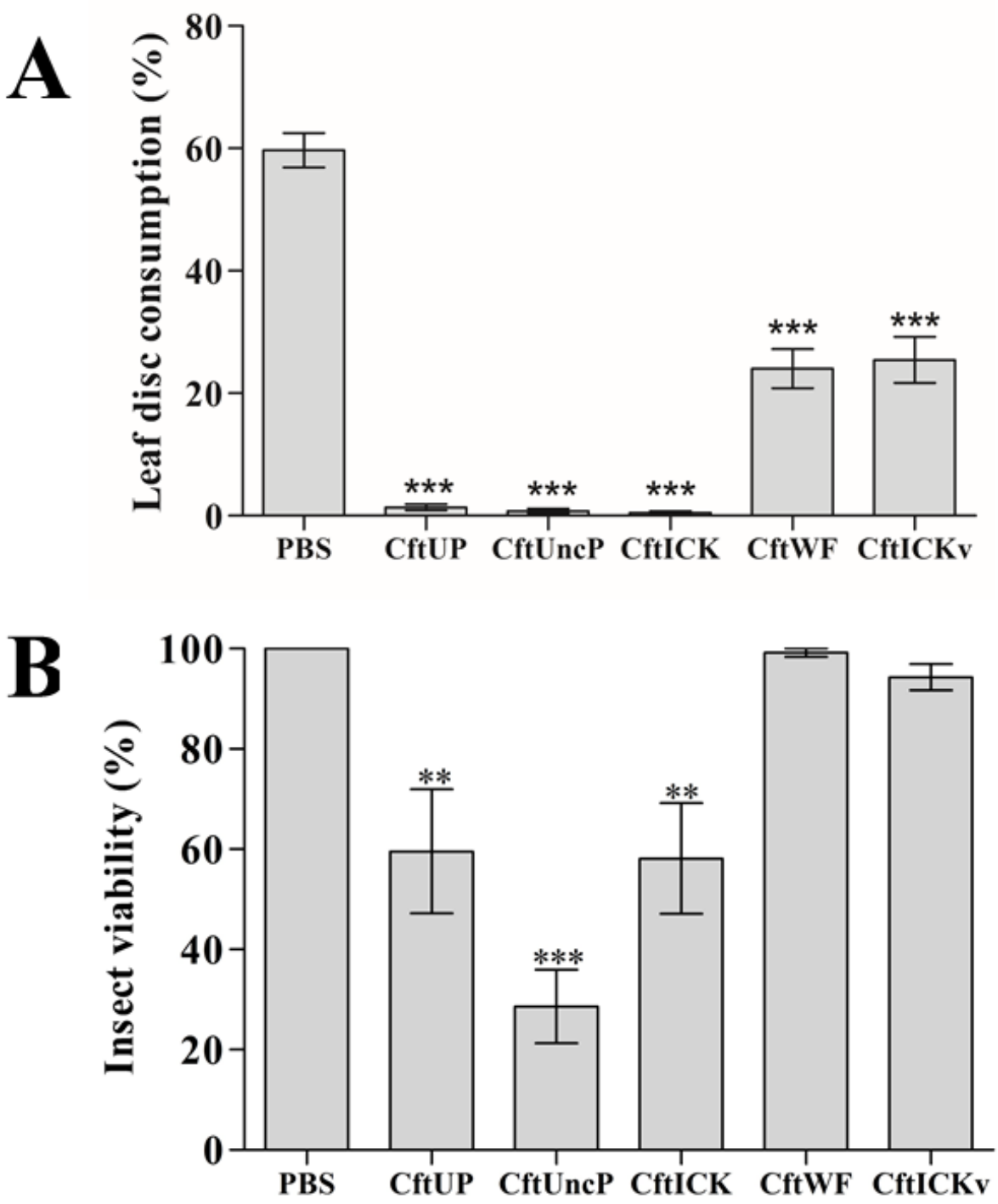


Figure 5. Insect viability and leaf ingestion by *D. saccharalis* neonate fed with sugarcane leaves covered by 100 µg mL^{-1} teratocyte derived peptides. A- Index of leaf consumption per larvae in three days. **B-** Neonates viability fed with sugar can leaves covered with the peptides. The bars represent ± standard error of the means. Asterisks, if present, above the bars indicate significant differences compared to control (PBS) by Dunnett's multiple comparison test (*P<0.05, **P<0.01, ***P<0.001).

4. Discussion

After parasitism, the number of hemocytes, an important immune-suppressive feature in host hemocoel, is often reduced to allow parasitoid immature development (Ibrahim and Kim 2006, Huang et al. 2009, Zhang et al. 2013, Wan et al. 2015, Trainor et al. 2021, Chapter III of this Thesis). For this, parasitoids use diverse sources of molecules, such as calix fluids, symbiotic polydnaviruses, venom, and teratocytes (Burke and Strand 2014).

At cellular level, the peptides CftUP, CftUncP and CftICK resulted in a decrease in hemocyte encapsulation capability. Encapsulation or nodulation, term vary accord the size of the invader, is the isolation and neutralization of invading bodies by covering them with layers of hemocytes that are partially disrupted for cell content release followed of melanisation mediated by PO (Dubovskiy et al. 2016). Probably, beyond CftUP, CftUncP and CftICK, *C. flavipes* uses other tools to regulate hemocytes behavior, derived from venom peptides and peptides produced after host cell infection with symbiotic polidnaviruses. This is the first time that individual peptides derived from teratocytes is tested against host hemocyte encapsulation, which requires complementary studies.

After testing five teratocyte-derived peptides, none of those altered the number of total circulating hemocytes (THC) in *D. saccharalis* larvae after 24 h of the injection of 10 µg of each peptide. Apparently, these peptides are not involved in THC reduction, but as most cellular regulation mechanisms of host regulation remain unclear, we should not discard the possible involvement these peptides in host regulation such THC when acting along with other peptides (Teng et al. 2016).

Humoral immune response represents another level of defense in insects against stressors, including bacteria, fungi and parasitoids. When an invading body access the inner part of an insect, the PO cascade is triggered to start an enzymatic conversion of hemocyte-bound prophenoloxidase (proPO) into the active form PO (Jiang et al. 2010, Whitten and Coates 2017). A known mechanism of inhibition of the activation of proPO by parasitoids is the release of serine protease inhibitors (serpins). Serpins reactive site targets an exposed loop near the carboxyl-terminal end of the serpin sequence and occupies the proteinase active site preventing the conversion of proPO into PO (Meekins et al. 2017). Another mechanism for inhibition of proteases involved in the activation of

pro-PO is also described in teratocytes, the trypsin inhibitor-like proteins (Gu et al. 2019). The peptides we tested showed no interference on the PO activity, suggesting the lack of involvement of them with PO inhibition or with the inhibition of the PO-activating cascade. Probably, *C. flavipes* account with serpins as main factors to disrupt proPO activation, considering that serpins are highly expressed after teratocytes release (Chapter III of this thesys).

The tested peptide CftICK is an inhibitor cysteine knot protein (ICK), also known as knottins. This is a family of ultra-stable miniproteins characterized by forming an intramolecular knot by the presence of at least three interwoven disulfide bridges (Postic et al. 2018). The recruitment of ICKs for host regulation by parasitoids was previously described (Parkinson et al. 2004), but, so far, their function on host regulation context were not elucidated. We found that ICKs have a special function in disrupting hemocyte nodulation. The properties of ICKs also allow their use as scaffolds for the engineering of pharmaceutical (Ackerman et al. 2014, Kintzing et al. 2016, Postic et al. 2018) and our data evidences their inhibiting property in cellular immune cells.

Teratocyte proteins characterized from parasitoid wasps, even still poorly studied, are known as a vast source of molecules with distinct functions (Strand 2014, Ali et al. 2015; Gao et al. 2016). The projection of teratocyte-derived molecules has been recently refreshed as an attractive alternative to Bt toxins for insect pest management (Merlin et al. 2021). In the current study, a functional analysis of five identified molecules from *C. flavipes* teratocytes was performed focusing on the immune system disruption activity and insecticide potential on *D. saccharalis*. Note that in a field situation, the target of transgenic toxins is the neonates, thus we tested the insecticide activity on recently hatched larvae. Further, we tested the immune system in the same age that in general *D. saccharalis* is parasitized by *C. flavipes.*

We observed a significant difference in lethality of CftUP, CftUncP and CftICK, all ICK-like proteins, compared to the control treatment. This is the first time that such peptides are tested for their insecticidal activity. Nevertheless, our study agrees with previous hypothesis that teratocytes are a potential rich source of potential pesticides (Maiti et al. 2003, Basio et al. 2005, Rossi et al. 2012, Merlin et al. 2020).

Koinobiont endoparasitoid wasps use a rich arsenal in order to control, but not immediately killing, their hosts. Our results increase the knowledge of teratocyte-derived functions, particularly on the disruption of hemocyte nodulation by teratocyte-derived ICKs peptides. We also found that an interesting pesticide potential on the same ICKs, which deserves more investigations to uncover their modes of action. Finally, our findings highlight that parasitoid host regulation molecules lack on characterization and further biotechnological exploitation, once such molecules are a diverse cocktail of novel peptides.

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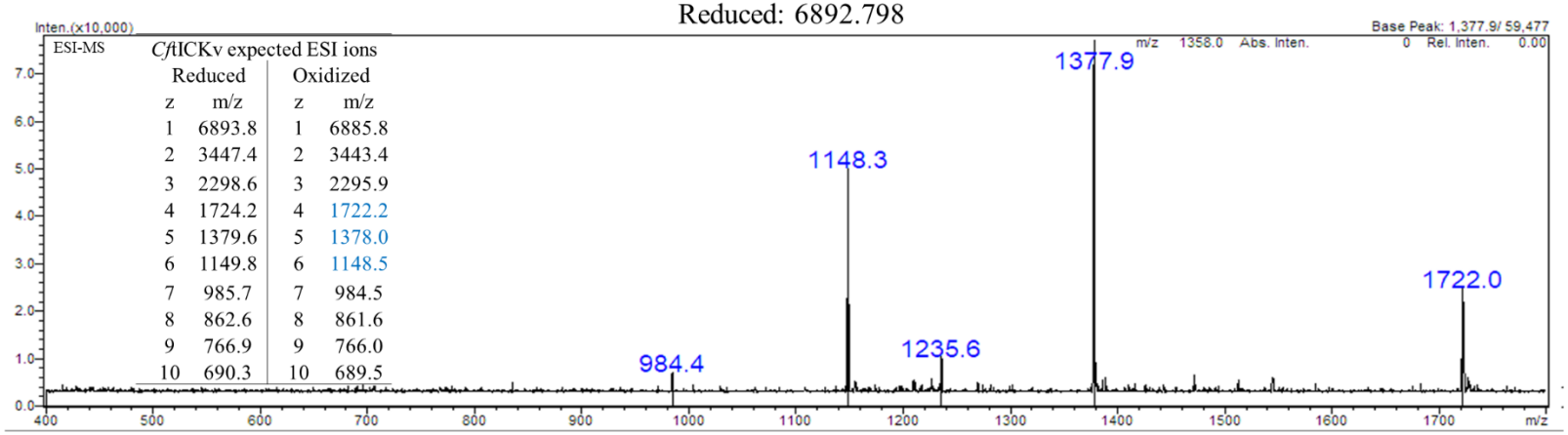
Supplementary data

*Cft*ICKv

AVYDYACYKKGCSKIQGRCSTNADCCDGYQCHPQTA
SWQVVQKNGCYPINPVPCSQLQQKNV
8 Cys
62 Aas
Oxidized: 6884.798
Reduced: 6892.798



*Cft*ICKv expected ESI ions

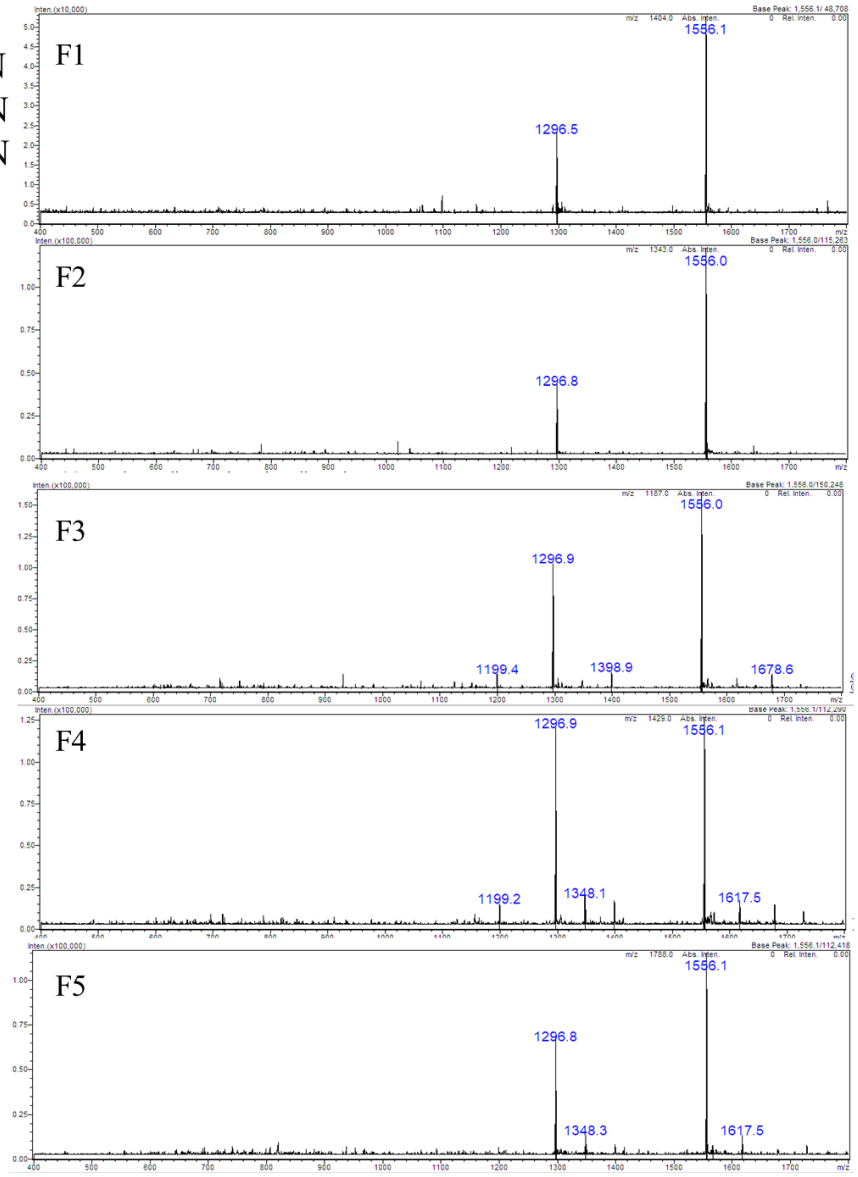
Reduced		Oxidized	
z	m/z	z	m/z
1	6893.8	1	6885.8
2	3447.4	2	3443.4
3	2298.6	3	2295.9
4	1724.2	4	1722.2
5	1379.6	5	1378.0
6	1149.8	6	1148.5
7	985.7	7	984.5
8	862.6	8	861.6
9	766.9	9	766.0
10	690.3	10	689.5

Supplementary Figure 1: RP-HPLC purified recombinant *Cft*ICKv detected on ESI. Expected ion masses were compared with detected ionized mass. Observed masses are all within 0.5 Da of the expected value.

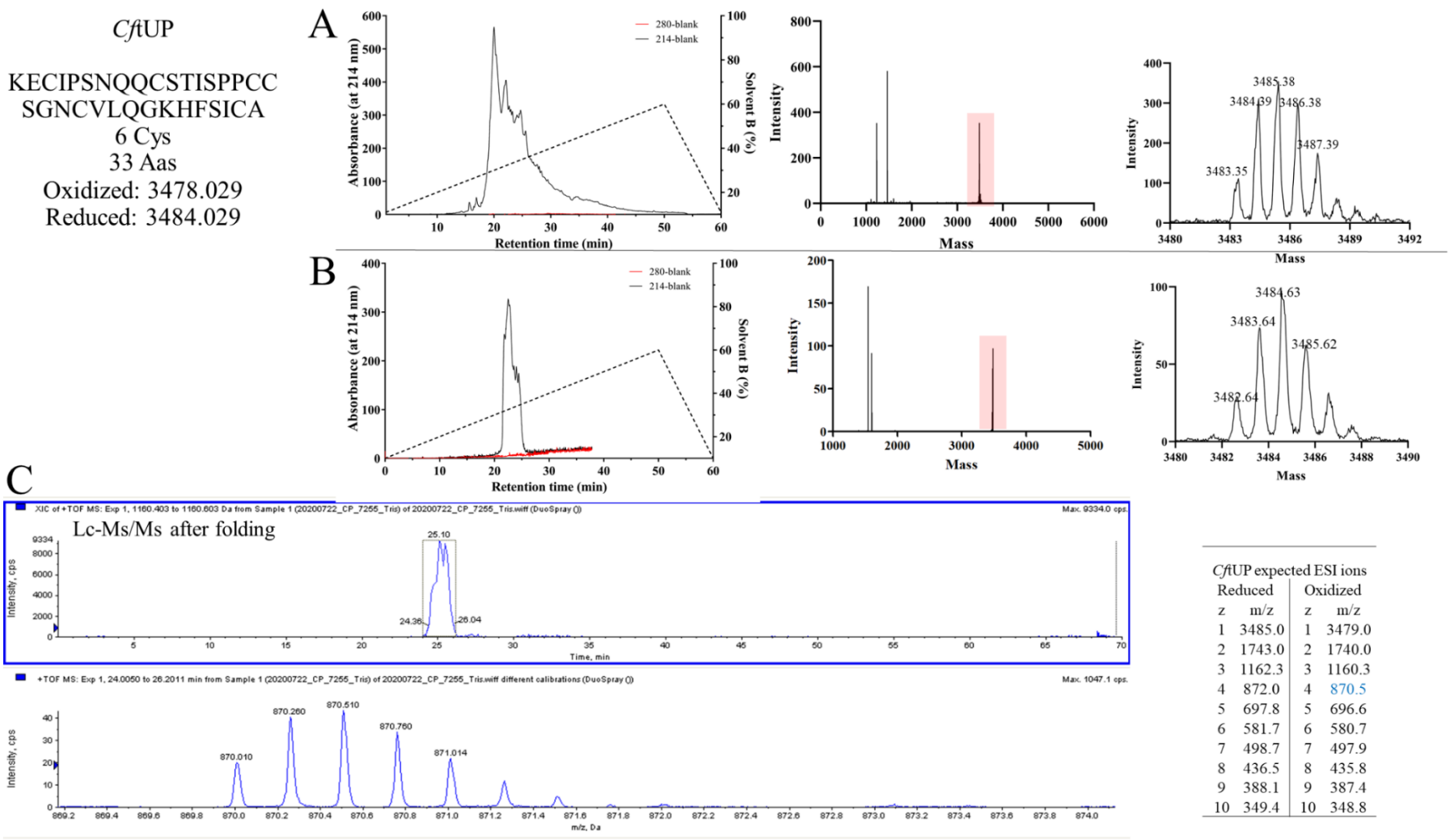
*Cft*WF
GDCPPFNNPQGETVPEGKTFDKN
CVQYTCKDGGYFATGCSESRCQN
QTGSTDYDFSLSFPECCPRPICPEN
8 Cys
70 Aas
Oxidized: 7775.474
Reduced: 7783.474

*Cft*WF expected ESI ions

Reduced		Oxidized	
z	m/z	z	m/z
1	7784.5	1	7776.5
2	3892.7	2	3888.7
3	2595.5	3	2592.8
4	1946.9	4	1944.9
5	1557.7	5	1556.1
6	1298.2	6	1296.9
7	1112.9	7	1111.8
8	973.9	8	972.9
9	865.8	9	864.9
10	779.3	10	778.5



Supplementary Figure 2: RP-HPLC purified recombinant *Cft*WF detected on ESI. Expected ion masses were compared with detected ionized mass. Observed masses are all within 0.5 Da of the expected value. The saturated RP-HPLC column generated an uneven peak collected as five fractions, all of them with the same mass.

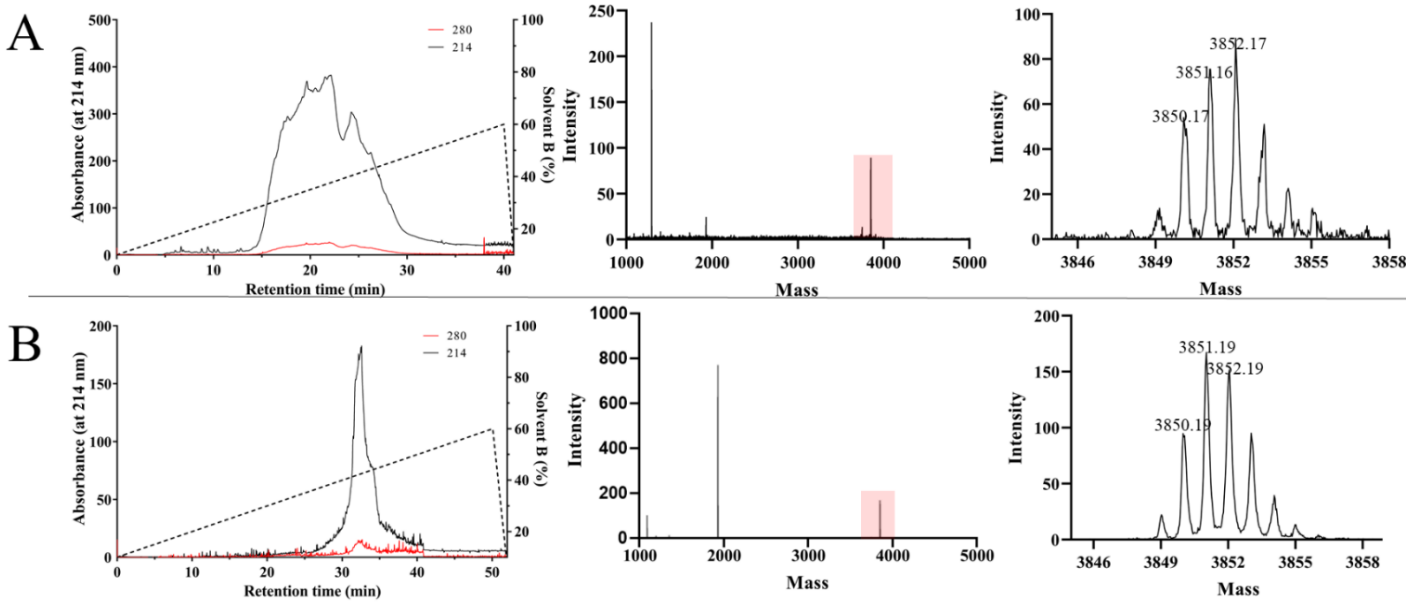


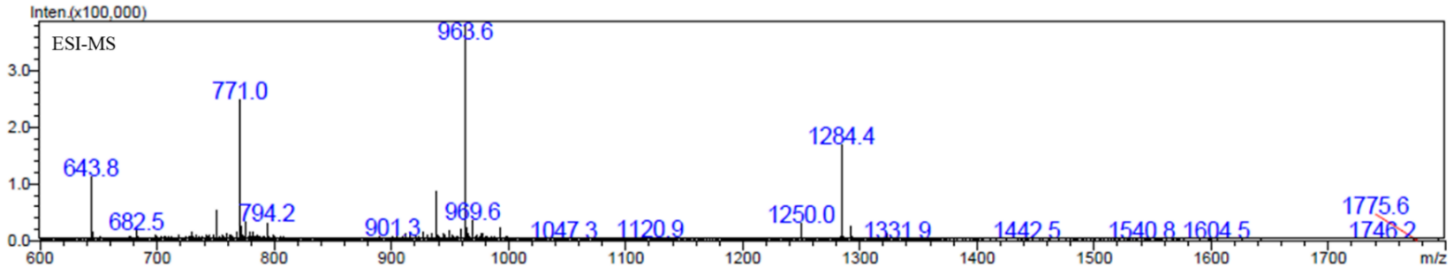
*Cft*UP expected ESI ions			
Reduced		Oxidized	
z	m/z	z	m/z
1	3485.0	1	3479.0
2	1743.0	2	1740.0
3	1162.3	3	1160.3
4	872.0	4	870.5
5	697.8	5	696.6
6	581.7	6	580.7
7	498.7	7	497.9
8	436.5	8	435.8
9	388.1	9	387.4
10	349.4	10	348.8

Supplementary Figure 3: Synthetic *Cft*UP. A and **B**- First and second batch of purification and verification of reduced monoisotopic mas by MALDI-TOF-TOF RP-HPLC. **C**- LC-MS-MS of purified and oxidized synthetic product, confirming the mass by the ionized fragment $M+4H^{+}$**.** Observed masses are all within 0.5 Da of the expected value.

CftICK

DGCLTYDSLCSTMYDKCCT
SMKCKGFFFGYCKSI
6 Cys
34 Aas
Oxidized 3844.521
Reduced 3850.521





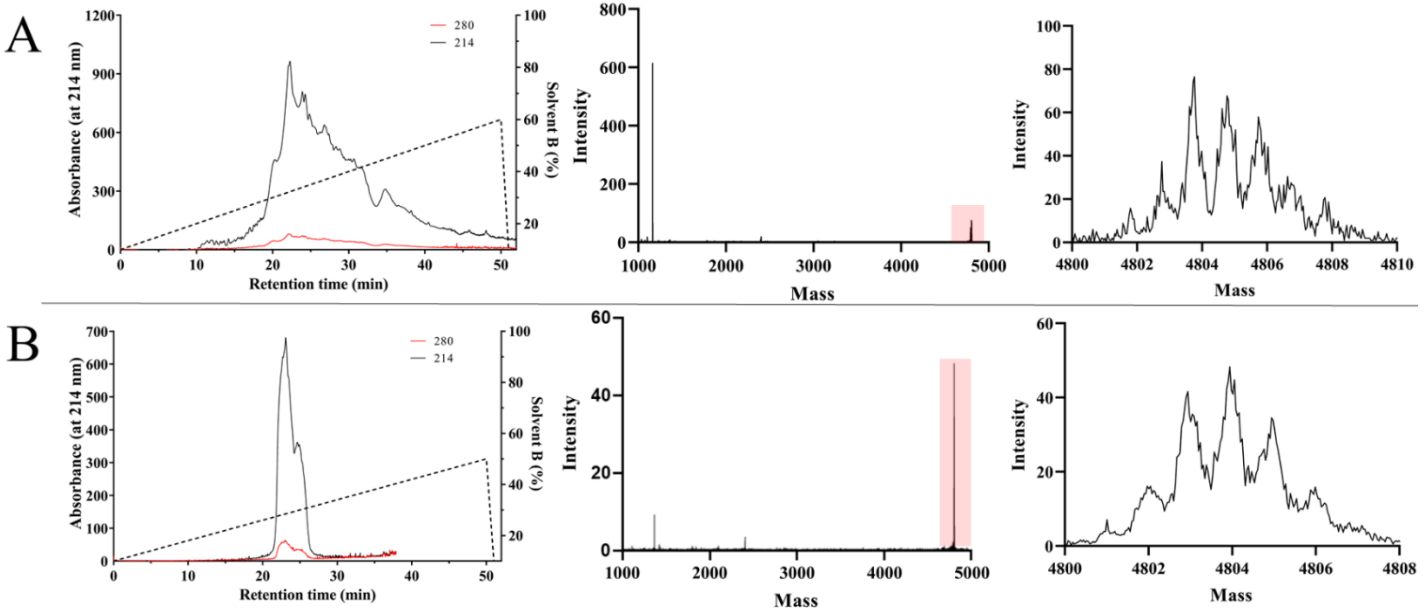
*Cft*ICK expected ESI ions

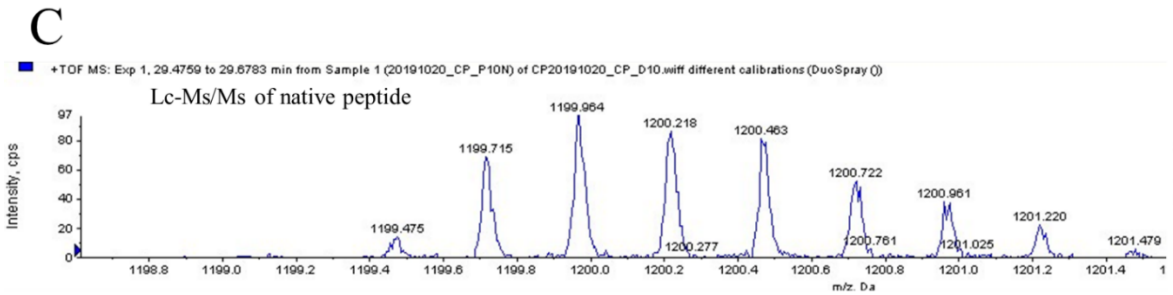
Reduced		Oxidized	
z	m/z	z	m/z
1	3851.5	1	3845.5
2	1926.3	2	1923.3
3	1284.5	3	1282.5
4	963.6	4	962.1
5	771.1	5	769.9
6	642.8	6	641.8
7	551.1	7	550.2
8	482.3	8	481.6
9	428.8	9	428.2
10	386.1	10	385.5

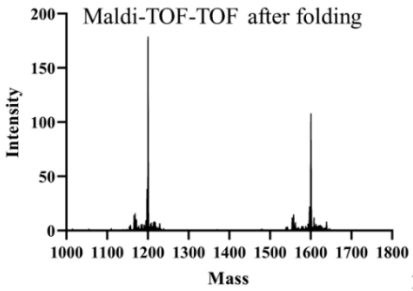
Supplementary Figure 4: Synthetic *Cft*ICK. A and B- First and second batch of purification and verification of monoisotopic mas by MALDI-TOF-TOF RP-HPLC. **C**- ESI of purified synthetic product, confirming the reduced mass ($M+3H^+$, $M+4H^+$, $M+5H^+$ and $M+6H^+$**.** Majority observed masses are all within 0.5 Da of the expected value.

*Cft*UncP

DICSHLGYACADDN
ECCDKFCKGLTPTTY
GICNKKPYTYRQY
6 Cys
42 Aas
Oxidized: 4797.417
Reduced: 4803.417



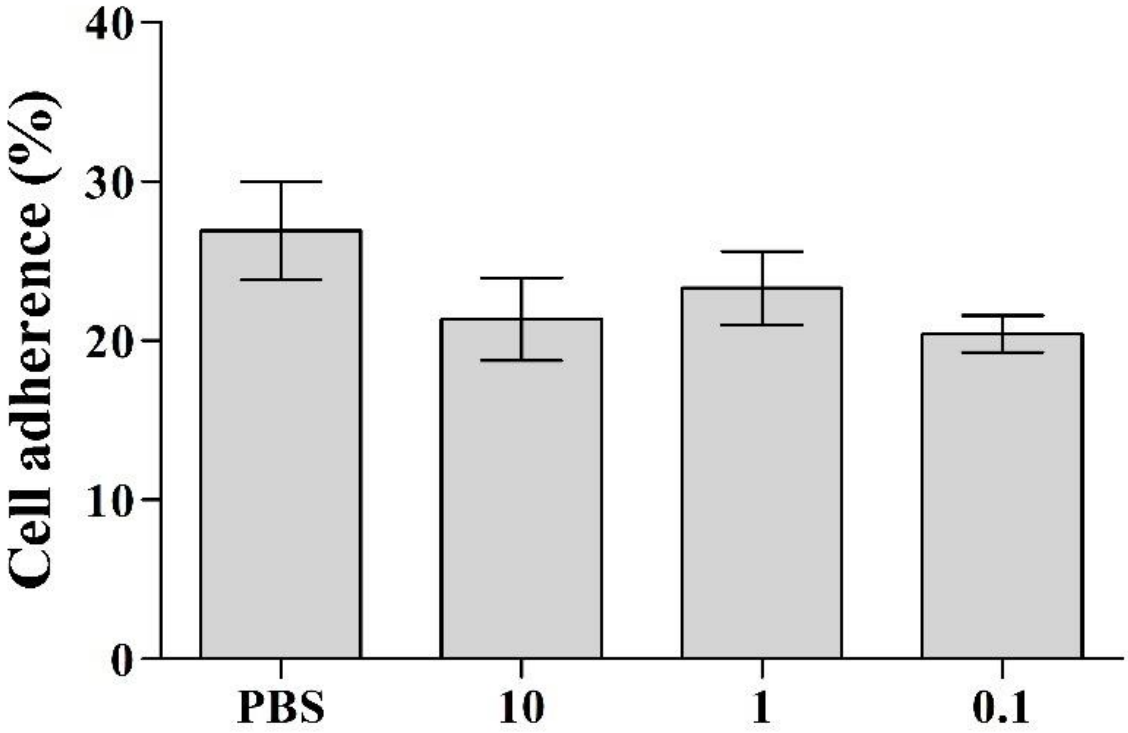




*Cft*UncP expected ESI ions

Reduced		Oxidized	
z	m/z	z	m/z
1	4804.4	1	4798.4
2	2402.7	2	2399.7
3	1602.1	3	1600.1
4	1201.9	4	1200.4
5	961.7	5	960.5
6	801.6	6	800.6
7	687.2	7	686.3
8	601.4	8	600.7
9	534.7	9	534.0
10	481.3	10	480.7

Supplementary Figure 5: Synthetic *Cft*UncP. A and B- First and second batch of purification and verification of monoisotopic mas by MALDI-TOF-TOF RP-HPLC. **C**- LC-MS-MS of native peptide followed by MALDI-TOF-TOF of oxidized peptide, confirming the correct mass by $M+3H^+$ and $M+4H^+$. Observed masses are all within 0.5 Da of the expected value.



Supplementary Figure 6. Influence of *Cft*ICKv on *Diatraea saccharalis* hemocyte adherence capability (%) quantified by the area of hemocytes adhered to a glass surface (covered area). Bars represent means ± standard errors. Asterisks, if present, above the bars indicate significant (*P < 0.05, **P < 0.01, ***P<0.001, Tukey test).

Chapter VI - Final Regards

Exploring and validating the biotechnological potential of molecules from distinct sources require extensive research. As discussed in the previous chapters, parasitoids use distinct tools to alter the physiology of their hosts. Despite several parasitoid species have their venom or teratocytes molecules described, the knowledge about individual functions of those peptides is still scarce. Because parasitoid-derived proteins may be related with immune suppression and also alter other physiological issues in the host, we hypothesized that peptides from a parasitoid species could present key roles in host immune regulation as well acting as pesticides after being ingested by an insect pest.

Herein, we demonstrated robust proteotranscriptomic approaches for identifying and testing molecules produced by the endoparasitoid *Cotesia flavipes* Cameron, 1891 (Hymenoptera: Braconidae), a massively used biological control agent for suppressing populations of sugarcane borer *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae) in Brazilian sugarcane fields.

We explored the diversity of peptides derived mainly from teratocytes and venom from *C. flavipes* and the methodologies performed in this thesis were reliable for the identification of parasitism-related peptides. In addition, we performed biological assays to validate the hypothesis of the selected peptides be acting over the immune system of *D. saccharalis* (humoral and cellular assays) as well their potential as pesticides after being ingested by *D. saccharalis* larvae.

Six peptides were selected for further immune functional and biotechnological analysis. Among those, one (Cf4) from venom apparatus and five (CftUP, CftUncP, CftICK, CftICKv and CfWF) from teratocytes. The criteria for peptide selection was peptides size (< 40 amino acid residues for synthesis) and number of cysteine residues in the peptides (≥ 6). Following, we produced these peptides using heterologous expression or solid phase synthesis.

We verified that the venom peptide (Cf4) and three teratocyte peptides (CftUP, CftUnkP and CftICK) disrupted the hemocyte nodulation capability from *D. saccharalis*. Four peptides (Cf4, CftUP, CftUnkP and CftICK) reduced *D. saccharalis* neonate viability after ingestion. Thus, we consider the peptides Cf4,

CftUP, CftUnkP and CftICK for further investigation and validation as insecticidal molecules.

Results presented in this thesis are important to validate parasitoids as a potential source of new pesticides, opening a new venue for further proteotranscriptome-based investigations aiming the identification of new peptides with agricultural purposes. Thus, it is expected that this thesis serves as a model workflow for subsequent investigations and, from it, novel and environmentally safe modes of action may become available for use in pest management in the near future.