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Functional and molecular analysis of venom produced by the ectoparasitoid *Bracon nigricans*

Ph.D. Dissertation
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Abstract

Parasitoid wasps have evolved a variety of strategies to regulate the physiology of their hosts in order to regulate the host's immunity and physiology for the interest of the development of its offspring. The venom of parasitic Hymenoptera, one of the major actors in the host regulation, has the potencial for developing novel environmentally safe insect control strategies and it is a blend of biologically, pharmacologically and physiologically complex mixture of peptides, amines, proteinaceous and nonproteinaceaous compounds). The neurotoxicity of the components present in the venom of *Bracon* species stimulated considerable research interests in this area, with the aim of isolating new agrochemical leads.

However, the characterization of venom produced by *Bracon* species is restricted to *B. hebetor*, *B. brevicornis* and *B. gelechie*. In *B. hebetor*, paralytic activity is mediated by three partially characterized proteins that presynaptically block glutaminergic transmission , and other unknow factors seem to affect host endocrine balance and metabolic activity.

In this work the interaction between ectoparasitoids and their hosts has been analyzed in the *B. nigricans* - *Spodoptera littoralis* model. The parasitism does not induce a rapid suppression of the host, even though the unaltered viability of hemocytes is associated with a suppression of their cellular division and/or production by haematopoietic organs. However, clear signs of a more subtle host regulation are evident, such as the reduction of CO_2 emission and the significant increase of hemolymph proteins and carbohydrates. The resulting enhancement of host nutritional suitability is associated with the success of progeny development and structural changes of the fat body cells, which interact with hemocytes and show signs of cell degeneration. The combined approaches of transcriptomic analysis on venom glands and proteomic study on crude venom resulted in the identification of several proteins, including a putative phospholipase A2 of 17 kDa. This

protein is probably the main toxin involved in the paralytic activity of *B. nigricans* venom. Additionally, a chromatographic fraction containing a protein band of about 17 kDa induces, when injected, a temporary paralysis in *S. littoralis* larvae. The identification of this protein and the production of recombinant phospholipase A2 will provide explanations on the functional role played by this bioactive component in the host regulation process.

Finally, as required by the PhD program, I have spent a period abroad where I studied the effects of silver nanoparticles against the red clover root borer, *Hylastinus obscurus* (Marsham) (Coleoptera: Curculionidae). Bioassays with silver nanoparticles showed that nanoparticles act as a feeding deterrent.

CHAPTER 1: Introduction

1.1 Insects

Insects (Insecta LINNAEUS, 1758) are a class of organisms belonging to the phylum Arthropoda, which contains 28 living orders. There are more species of insects than all other land animals put together and are believed to be among the oldest settlers of the land (Figure 1.1).

The heterogeneity in morphology, anatomy, biology and ethology has given them, for over 300 million years, a prominent role in the colonization of the globe. Insects have a remarkable ability to compete and thanks to the development of specific adaptations they are able to use the most varied food substrates and to live in extreme environmental conditions.

For these reasons, insects are considered the group of organisms at the top of the evolutionary ladder. Many insects attack other living organisms and, at the same time, are the target of many natural enemies and pathogens. On the base of the relationships that they establish with other organisms exist countless mechanisms of interaction, often very significant at economic, health and social level.

In fact, the establishment of such associations is often mediated by the development of virulence factors and/or mechanisms of resistance, selected by long coevolutionary processes, lasted hundreds of millions of years. The antagonistic associations between insects and other arthropod species are considered among the most interesting, in particular insect/insect interactions, characterized by different modes and various levels of specialization.

In insect's world, switching from relatively simple trophic interactions such as those that exist between insect predators and their victims, to the complex interactions that exist between physiological and biological insect parasitoids and their hosts.

The study and manipulation of the associations between insects and other organisms has a long and established tradition, due to the variety of potential applications of a detailed understanding of these interactions. In particular, in recent decades, the advancement of knowledge in the field of basic and applied entomology has enabled the implementation of control strategies integrated with low environmental and toxicological impact (Viggiani, 1997). Moreover, the growing information accumulated in the fields of genomics and post-genomics has led to the development of new methods of controlling insects harmful to man and its products.

In particular the study of molecular interactions "plant-pest-entomophagous" has greatly expanded the knowledge base for the development of new sustainable strategies for plant protection.

Through the investigation of these antagonistic relationships and mechanisms that regulate them, as well as the advancement of knowledge on the molecular level of insects's physiology, development, reproduction and behavior is already been possible to draw biotechnology of insect pests control based on use/manipulation of biological agents (Beckage and Gelman, 2004; Pennacchio *et al*., 2012).

It's easy to predict that, as they will continue their studies, the development of these technologies will receive a pulse increasing, with almost unlimited application perspectives.

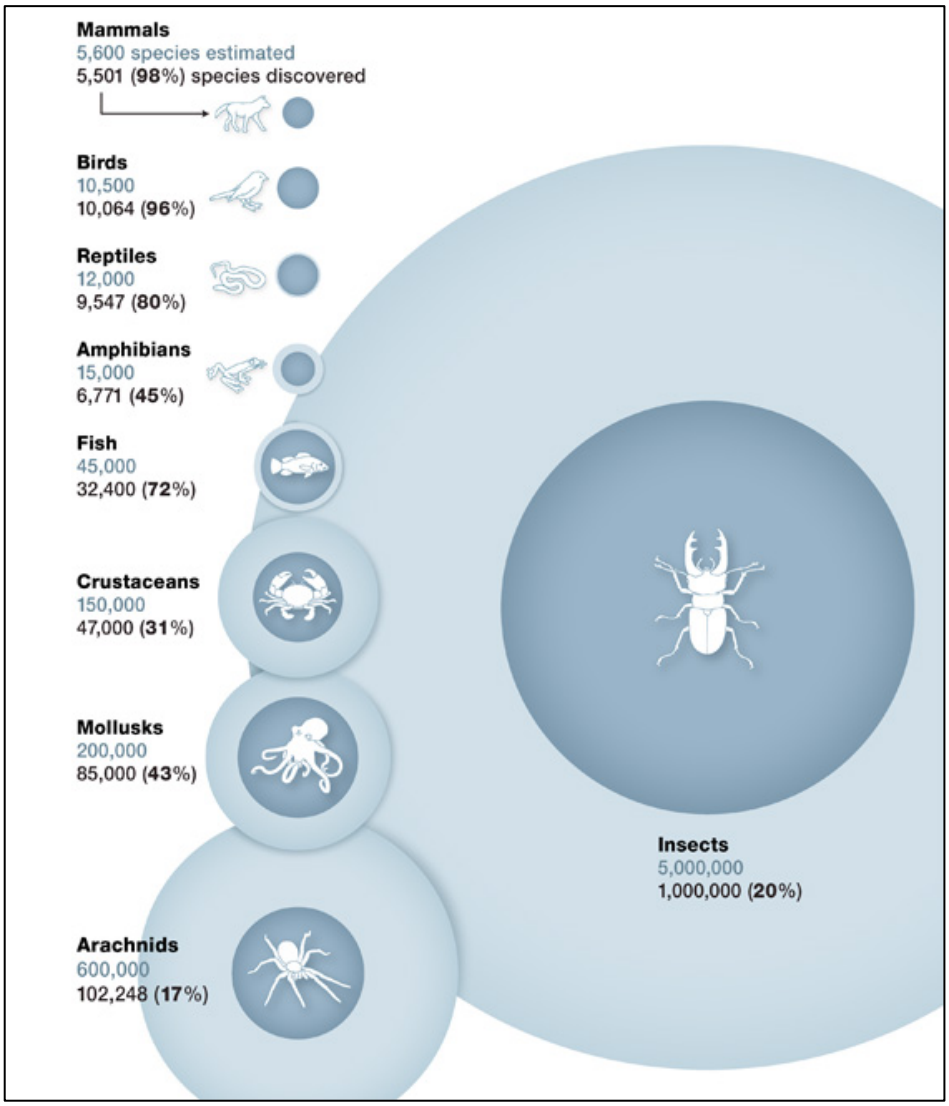


Figure 1.1. Illustration about the number of insect (estimated and discovered) respect the rest of animals in the world. From IUCN: Arthur D. Chapman, Australian Biodiversity Information Services.

1.2 Entomophagous insects: the parasitoids

With more than 150,000 species, parasitoids are entomophagous insects, that exploit other arthropods from which they take benefit (nourishment, protection) causing their biological damage. Parasitoidism is a transitional form between the predator and the parasite forms. In fact, parasitoids have some prerogatives of the parasite, as they are devoid of independent life during juvenile stages and depend from the host to which they are more or less closely related to anatomical and physiological relationships while the

adult leads free life and can lay their eggs in most individuals of the same species or, in some cases, different species (Pennacchio and Strand, 2006).

The death of the host happens before the host reproduces and, consequently, its fitness directly depends on its ability to escape parasitism or to resist it. In turn, as parasitoids cannot succeed their development without hosts, their fitness directly depends on parasitism success (Poirié *et al.*, 2009).

Although several organisms have developed this type of adaptive strategy, it is particularly common in holometabolous insects (Diptera, Coleoptera, Lepidoptera, Trichoptera, Neuroptera and Strepsiptera) but more than 80% of the described species belong to the order Hymenoptera (Quicke, 1997).

Parasitoid wasps have evolved a wide spectrum of interactions with their host (Godfray, 1994, Pennacchio and Strand, 2006). Different interactions have been classified in relation to their biology.

From the simple solitary (at most one offspring is able to complete development to adult on or in a host) and gregarious parasitoid species (more than one offspring can successfully complete development on or in a host) to the koinobiont and idiobiont parasitoids which emphasized the developmental strategy of the parasitoid. In a broad sense, Askew and Shaw (1986) categorized parasitoids as koinobiont and idiobiont parasitoids due to their developmental behaviours (Table 1.1).

Table 1.1. From Pennacchio *et al*., 2014.

Different host strategies adopted by zoophagous insects			
	Exploited hosts	Host regulation	Nutritional interactions
Predators	- Several hosts, generally consumed by all stages. - Host captured and used.	- None	- Limited, if any. - Breakdown of host tissues.
Parasitoids *idiobiont*	- Single hosts consumed by juveniles. - Most species are ectophagous. - Include egg, pupal and larval (concealed) parasitoids.	- Development blocked. - Metabolism reduced. - Immunosuppression to favor food uptake and use.	- Nutrient mobilization from host tissues. - Breakdown of host tissues.
Parasitoids *koinobiont*	- Single hosts. - Most species are endophagous of larvae and adults. - Include a few ectophagous species of exposed larvae.	- Host continues to develop. - Immunosuppression to prevent encapsulation. - Development and reproductive alterations. - Endocrine manipulation.	- Nutrient availability manipulated and synchronized to meet parasitoid needs. - Parasitoid larvae have specialized pathways of nutrient absorption.

In the case of koinobiont the venom of the wasp determines a transient paralysis and the host continues its development until the complete maturation of the parasitoid's progeny. In fact, after oviposition, koinobiont parasitoid offspring allows the development of the hosts and eventually the host is killed when the parasitoid emerge as an adult. The majority of koinobionts are endoparasitoids characterized by a longer larval stages that develops inside the host body's cavity; the adults generally have a short life and produce small eggs. The egg hatches immediately but undergoes a quiescent period while the host grows to an appropriate size and stage. Koinobionts exercise

some control over the development of their hosts (Vinson and Iwantsch, 1980; Pennacchio *et al*., 2014), and because they are closely associated with the life cycles of their hosts they have limited host ranges. Although oviposition is usually stage-specific, koinobionts can attack various stages of pre-imaginal development of the host. In general the "host range" is extremely limited, the venom acts on a small number of hosts, often it is a single species due to physiological and biological interactions and coevolution established between the host and the parasitoid (Pennacchio and Strand, 2006).

In contrast, an idiobiont completely paralyzed the host by the action of the wasp venom and the host is unable to defend himself and to complete its development (Pennacchio *et al*., 2014). The majority of idiobionts are ectoparassitoids characterized by a wide host-range showing larval stages extremely voracious and short. Ectoparasitoids usually attack host larvae that are half grown or larger which are contained in cells, leaf rolls, or burrows or are beneath a web or other covering. The adult stage is long and characterized by the production of a few large eggs. The oviposition usually takes place in the life stage of the host, that can provide a complete and satisfactory supply to the parasitoid offspring (last instar larvae and pupae). Idiobiont parasitoids paralyze permanently its host immediately at oviposition and shortly thereafter the host dies.

Both in idiobiont and koinobiont, the success of parasitism depends on a number of factors that induce physiological and metabolic alterations and allow the parasitoids to evade the immune system of the host (Strand and Pech, 1995; Strand, 2012). This is particularly true for koinobionts, capable of a fine regulation of the endocrine secretions, metabolism, reproduction and development of the host (Vinson *et al*., 2011; Beckage and Gelman, 2004; Pennacchio and Strand, 2006; Pennacchio *et al*., 2014).

The overall picture of the effects produced in the host by the parasitoid is defined by the term “host regulation” (Vinson and Iwantsch, 1980).

1.3 Host regulation by ectoparasitoids

Parasitoid wasps have evolved a variety of strategies to regulate the physiology of their hosts in order to enhance the success of parasitism (Pennacchio and Strand , 2006). The diversity of these strategies reflects the intense radiation process of Hymenoptera, which starting from the ancestral basal condition of ectophagy has generated a wealth of different adaptive strategies to exploit at the best different food sources, reducing the mortality risks. This has promoted the development of endophagous life habits, which evolved at different times and independently in separate evolutionary lineages (Gauld,1988; Whitfield, 1992; 1998).

The diversification process of parasitic Hymenoptera and the resulting colonization of a number of different ecological niches has been determined by the parallel diversification of the molecular “toolkit” used by the ovipositing females (which include venom and different reproductive secretions) to regulate the host physiology (Pennacchio and Strand, 2006).

While for endophagous wasps, in a limited number of model systems, mostly in the superfamily Ichneumonoidea, there is a fairly large number of studies addressing this research issue, we have a very limited and scattered information for ectophagous wasps.

Ectoparasitic wasps are important natural enemies of a vast array of insects that can achieve pest status in agricultural systems. Several species are important biological control agents used in integrated pest management programs relying on augmentative and/or inoculative release strategies (Danneels *et al*., 2010).

These parasitoids also produce an incredible and unutilized pharmacopoeia of venoms that may serve as leads for developing new classes of synthetic chemical insecticides (Danneels *et al*., 2010).

Many idiobionts are ectoparasitoids which often paralyze their hosts by injecting venom secretions into hosts and whose offspring feed by using their mouthparts to pierce the host's cuticle (Pennacchio *et al*., 2014). Both clotting of host hemolymph and melanization induced by wounding (Lemaitre and Hoffmann, 2007; Rivers *et al*., 2002) can interfere with food uptake and use by ectoparasitoids (Strand and Pech , 1995). However, many ectoparasitoids suppress these host defense responses through factors present in venom or salivary secretions produced by feeding wasp larvae (Richards and Edwards , 2002). Their venoms have evolved into different mechanisms for manipulating host immunity, physiology and behavior in such a way that enhance development of the parasitoid young (Danneels *et al*., 2014). The venoms of several ectoparasitoids contain endocrine disruptors that reprogram the development of the host to favor that of the parasitoid; these include paralytic factors as well as developmental arrestants that inhibit molting of the host so that the parasitoids are not dislodged from the host's external surface prematurely (Beckage and Gelman, 2004).

Paralysis may be transient, occurring immediately post-parasitization, or irreversible, depending on the species. In general, host larvae attacked by ectophagous braconids are usually permanently and completely paralyzed.

To date, several information available for the idiobiont ectophagous wasps is relative to the genus *Bracon* (Hymenoptera, Braconidae). The neurotoxicity of the molecular components present in the venom of *Bracon* species stimulated a considerable research interests in this area, with the aim of isolating new agrochemical leads (Weaver *et al*., 2001).

The venom of the ectoparasitic braconids *B. hebetor*, *B. brevicornis*, and *B. gelechiae* induces potent paralyzing effects in their hosts (Beard, 1978). It is

estimated that in larvae of the greater wax moth, *Galleria mellonella*, the venom of *B. hebetor* causes complete and permanent paralysis at the level of one part *B. hebetor* venom to 200 million parts host hemolymph (Weaver *et al*., 2001). In *B. hebetor*, paralytic activity is mediated by three partially characterized proteins that presynaptically block glutaminergic transmission, and other unknow factors seem to affect host endocrine balance and metabolic activity (Chanda *et al*., 2002; Masler and Kovaleva, 1999). Multiple toxins in these species have been identified, including high molecular weight molecules, which appear to be hexamerins, as well as low molecular weight paralytic components (Beckage and Gelman, 2004).

The venom is produced by venom apparatus associated with reproductive organs of wasp females.

In general the venom apparatus consists of venom gland filaments, the reservoir and the primary duct. These structures have ectodermic origin and are dorsally placed in the metasoma; they show varying evolutionary advancements depending on the Hymenoptera group (Peydrò *et al*., 1996). The morphology of the venom apparatus presents some modifications depending on the type of parasitism of each parasitoid group, and also it offers some information about the biology of these parasitoids and the parasitoid-host relationships. Even though the venom apparatus is present in every female of Braconidae, the nature and function of the substances produced by it vary in accordance with its biology (Edson and Vinson, 1979). *B. nigricans* females have a completely spiral sculptured reservoir intima and the gland filaments are arranged around a distinct protruding structure at the base of the reservoir (Quicke *et al*., 1992) (Figure 1.2).

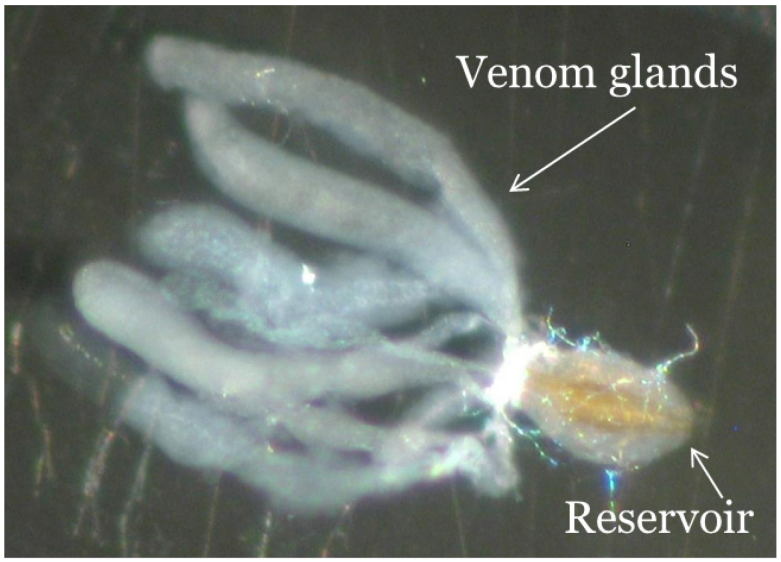


Figure 1.2. Venom apparatus of *B. nigricans* female.

In general, the role of the venom in ectoparassitoids induces permanent paralysis, developmental arrest of the host, regulation of metabolism and the inhibition of the host immune response (Nakamatsu and Tanaka, 2003).

1.4 Parasitism and immune system

Multicellular animals defend themselves against infectious organisms by two systems known as innate and acquired immunity. The innate immune system relies on germline encoded factors for recognition and killing of foreign invaders, whereas the acquired immune system produces receptors by somatic gene rearrangement that recognize specific antigens and that allow organisms to develop an immunological memory (Fearon, 1997).

Insects do not have and acquired immune system. Nevertheless they can count on an efficient innate immune system capable of recognizing *non-self* materials such as the eggs of a parasitoid. Initial defenses include the physical barriers of the integument or gut, clotting responses by hemolymph, and the production of various cytotoxic molecules at the site of wounding. (Lavine and Strand, 2002). The entrance in the haemocoel of pathogens and/or invading organisms causes an innate defense response that involves humoral

and cellular mechanisms. In fact, the immune system of insects is divided into humoral and cellular defense. Humoral defenses include the production of antimicrobial peptides (Meister *et al*., 2000; Lowenberger, 2001), reactive intermediates of oxygen or nitrogen (Bogdan *et al*., 2000; Vass and Nappi, 2001), and the complex enzymatic cascades responsible of hemolymph melanization and coagulation (Muta and Iwanaga, 1996; Gillespie *et al*., 1997). Cellular responses are instead mediated by hemocytes and include responses such as phagocytosis, nodulation and encapsulation (Strand and Pech, 1995; Schmidt *et al*., 2001; Rivers *et al*., 2002). Dividing the insect immune system into cellular and humoral responses is somewhat arbitrary, as many humoral factors affect hemocyte function and hemocytes are an important source of many humoral molecules. There also is considerable overlap between humoral and cellular defenses in processes like the recognition of foreign intruders. (Lavine and Strand, 2002). Insects produce several types of hemocytes that are traditionally identified using morphological, histochemical and functional characteristics (Gupta, 1985; Brehelin and Zachary, 1986). The formation of the capsule begins when a small number of hemocytes attacks the foreign body target, followed by other hemocytes that gradually forms multiple layers. Several studies have shown that, in some insects, a single class of hemocytes is involved in the formation of the capsule (Brehelin *et al*., 1975), while other studies indicate that encapsulation requires the cooperation of different morphotypes of hemocytes (Ratcliffe, 1993).

The most common types of hemocytes reported in the literature are prohemocytes (the progenitors of mature hemocytes), granular cells (granulocytes), plasmatocytes, spherule cells (spherulocytes), and oenocytoids. Immunity response of granulocytes and plasmatocytes involve their adherence to foreign surfaces or to other hemocytes leading to phagocytosis or encapsulation phenomena. On the other hand, non adherent

hemocyte types have poorly understood functions. In moths, the most abundant circulating hemocytes in hemolymph are granulocytes, responsible for phagocytosis, and plasmatocytes that forming capsules and nodules. The success of parasitism depends mainly on the suppression of the immune system of the host.

1.5 *B. nigricans* Szépligeti (Hymenoptera: Braconidae)

The Braconidae constitute one of the most species-rich families of insects. Although tropical faunas are still relatively poorly understood at the species level, most taxonomists in this group would agree that a rough, probably highly conservative, estimate of 40-50,000 species worldwide is reasonable as an extrapolation from the current described number of roughly 12,000 species. Among extant groups, the sister group of the Braconidae is the Ichneumonidae, an equally enormous group (Sharkey and Wahl, 1992; Quicke *et al*., 1999).

The family appears to date from early Cretaceous (assuming Eobracon is properly assigned to family - Rasnitsyn, 1983; Whitfield, 2002), diversifying extensively in the mid to late Cretaceous and early Tertiary, when flowering plants and their associated holometabolous herbivores, the main hosts for braconid parasitoids, radiated (Basibuyuk *et al*., 1999; Quicke *et al*., 1999; Belshaw *et al*., 2000; Whitfield, 2002).

They range in size from approximately 1 mm to 3-4 cm in length (not counting the ovipositor, which in some species can be several times as long as the body).

The vast majority of braconids are primary parasitoids of other insects, especially upon the larval stages of Coleoptera, Diptera and Lepidoptera but also including some hemimetabolus insects (aphids, Heteroptera, Embiidina).

Both external and internal parasitoids are common in the family, and the latter forms often display elaborate physiological adaptations for enhancement of

larval survival within host insects, including the co-option of endosymbiotic viruses for compromising host immune defenses (Stoltz and Vinson, 1979; Stoltz, 1986; Whitfield, 1990; Beckage, 1993, Stoltz and Whitfield, 1992; Whitfield, 2002; Whitfield and Asgari, 2003). Most braconids are primary endoparasitoids of Lepidoptera larvae, although most holometabolous groups may be attacked, e.g., Diptera, Coleoptera and other Hymenoptera. Some species attack spiders, and some are hyperparasitic. There are both solitary and gregarious species in the family, and some are ectoparasitic. Braconids have been used extensively in biological control with much success (Sharkey, 1993; Ghahari *et al*., 2006).

This is one of the major groups of insect parasitoids that includes a large number of species that are effective enough to exert a definite regulatory impact on the increase of numerous important plant pests.

During the years after the invasion of Western Palaearctic tomato crops by the South American tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), several indigenous generalist parasitoids have been recorded on this new host (Biondi *et al*., 2013 a. Among *Bracon* spp. recovered from South American tomato leafminer, *B. nigricans* Szépligeti (= *concolorans* Marshall; = *concolor* Thomson; = *mongolicus* Telenga) (Hymenoptera: Braconidae) is widely distributed in the Palaearctic region (Yu and van Actherberg, 2010). Available data on this species consist mainly of taxonomic information and its recovery in faunistic surveys (Zappalà *et al*., 2012) and recently a study on population dynamic related traits affected by biopesticides (Biondi *et al*., 2013b. The first record of *B. nigricans* on *T. absoluta*, that is, a new host parasitoid association, is from a survey of indigenous parasitoids attacking the invasive pest in in some of the regions of Southern Italy (Campania, Sardinia and Sicily) (Zappalà *et al*,. 2012). Subsequently, *B. nigricans* was reported in Jordan (Al-Jboory *et al*., 2012) and Spain (Urbaneja *et al*., 2012).

B.(*Habrobracon*) *nigricans* Szépligeti is an ectophagous parasitoid of lepidopteran larvae, exploited as biocontrol agent of the tomato leaf miner *T. absoluta* (Zappalà *et al*., 2012). Various indigenous species of parasitoids and predators feed on *T. absoluta* in the Mediterranean basin (Zappalà *et al*., 2013; Desneux *et al*., 2010; Gabarra and Arnó, 2010; Mollá *et al*., 2010; Loni *et al*., 2011). They are gradually getting adapted to the new pest and under laboratory conditions *B. nigricans* was able to reduce significantly *T. absoluta* populations not only owing to the parasitism activity but also thanks to non-reproductive host killing activity, namely, host-feeding and host-stinging behaviours (Ferracini *et al*., 2012; Biondi *et al*., 2013a) and will probably play a central role as limiting factors in the near future.

Idiobiont parasitoids like *B. nigricans* do not need to adapt to their host physiology and therefore they may switch more easily to new hosts (Askew and Shaw, 1986), providing, in some cases, a substantial control of insect pests, especially of leafminers (Godfray *et al.*, 1995; Urbaneja *et al.*, 2000).

Thanks to its polyphagous behaviour, *B. nigricans* attack a broad range of host species, including *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) under laboratory conditions.

This species was used as a model host to carry out the experiments in this thesis. The main reason for using *S. littoralis* is due to the larger size of the larvae compared with those of *T. absoluta* that allows a better manipulation for the success of the bioassays (see Figure1.3).

Figure 1.3. Difference in size of *S. littoralis* and *T. absoluta* larvae on tomato fruit.

The life cycle of the parasitoid lasts about 12-13 days. Females lay 3 to 20 eggs on each larva, after 48 h the eggs hatch and newly hatched larvae begin to feed on the body fluids. After about 3 days the larvae are ready to build the cocoon and after 7 days new adults emerge (Figure 1.4).

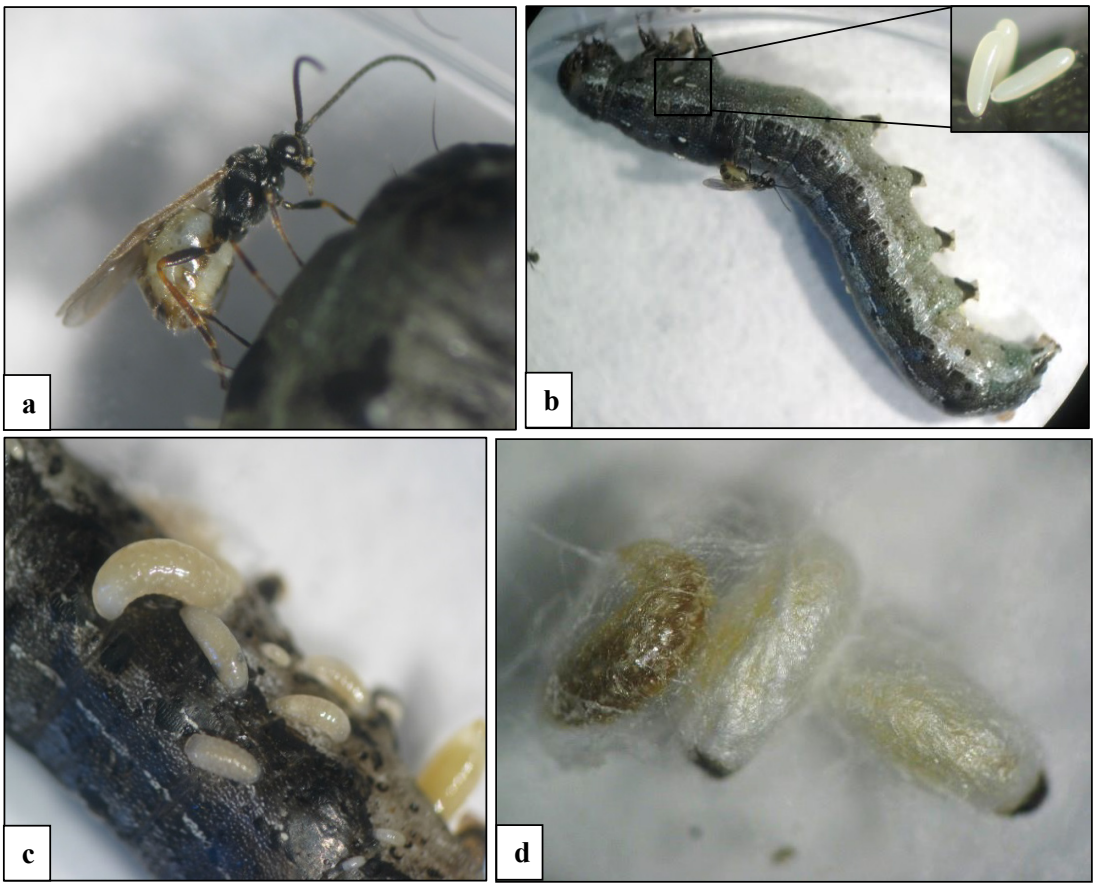


Figure 1.4. (a) *B. nigricans* female; (b) *S. littoralis* paralyzed larvawith a particular of parasitoid's eggs; (c) parasitoid larvae feed on host; (d) cocoons of parasitoid.

B. nigricans showed biological traits of an idiobiont ectoparasitoid. Female wasps demonstrated a good adaptability to a new host and behavioral plasticity in host use; as described by Biondi *et al*. (2013a) for *T. absoluta* an initial host paralysis was followed by intensive host examination leading to host-feeding, oviposition, or a host rejection.

B. nigricans females likely paralyzed 4th or 5th instar *S.littoralis* host larvae with the first ovipositor insertions, probably because of the injection of venom that causes irreversible paralysis in the larvae after few seconds, as other idiobionts and a few koinobionts do (Pennacchio and Strand, 2006). After initial paralysis, females intensively probe the host with their ovipositor before ovipositing and/or feeding on a host. Females fed either directly on the paralyzed host or inside the leaf mines (in case of leafminers) after building feeding tubes used to suck host tissues and hemolymph, that are a rich source of nutrients, like proteins, fats and sugars.

1.6 Bioinspired insecticides

With the advent of commercially avaible synthetic pesticides in the 1940s, chemicals became the major method of controlling agricultural, urban and medically important pests (Tedford *et al*., 2004). Insects in particular have become the target of chemical control programs because of their large, global populations and refractory response to other management means (Gentz *et al*., 2009). The compatibility of these chemical control options with biological control or integrated pest management (IPM) program is becoming paramount, since the use of these technologies in tandem is being increased in order to reduce the use of broad-spectrum pesticides (Batra, 1982) and to delay the onset of insecticide resistence (Broadhurst, 1998; Hoy, 1998).

Important characteristics for IPM-compatible and reduced risk pesticides include high selectivity for target pest, low non-target effects (especially on biological control agents), and short term environmental persistence (Gentz *et*

al., 2009). Reducing the use of pesticides is one of the main objectives in sustainable agriculture, widely pursued through the use of alternative products with low environmental impact and biological control agents.

Moreover, many insect pests have developed resistance to existing chemical insecticides and together with the de-registration of key insecticides due to perceived ecological and human health risks there is much interest in the development of new insecticidal compounds with novel modes of action (Cooper and Dobson, 2007; Tedford *et al*., 2004; King and Hardy, 2012). Many antagonistic associations in insects are sources of new natural compounds that can alter the growth, development, reproduction and the immune system of pests, and therefore, offer interesting opportunities for the development of new biological insecticides.

These last years, an increasing number of original peptides, proteins, and enzymes have been reported from the venoms of ichneumonid, braconid or eucoilid wasps (Digilio *et al*., 2000; Parkinson *et al*., 2001, 2002a-c, 2003, 2004; Asgari *et al*., 2003a; b; Dani *et al*., 2003; Moreau *et al*., 2004; Zhang *et al*., 2004; Labrosse *et al*., 2005b). These findings may constitute a new step in the investigation of several fundamental topics such as parasitoid- host physiological relationships, phylogeny of parasitoid Hymenoptera or biosynthesis and functional evolution of insect proteins. In addition, they represent an underestimated source of new molecules with potentials in the agronomic and biomedical sectors (Moreau and Guillot, 2005).

To date, just a few enzymatic activities of parasitoids venoms have been reported and there isn't an equal ratio in respect to the estimated number of species belonging to the group of parasitoid Hymenoptera (100,000-250,000) (Askew, 1971; Gauld,1986). This strikingly underlines our great ignorance of the enzymatic diversity of parasitoid venoms. Some characteristics may nevertheless be pointed out from the analysis of the few enzymes known to date (Moreau and Guillot, 2005).

Different parasitoid species could have evolved under specific selective pressures different venomous enzymatic mixtures, that may constitute a molecular testimony of the evolutionary history of parasitoid species, marked by highly specialized adaptations to their host range (Whitfield, 1998; Schmidt *et al*., 2001). The majority of known enzymes from venoms parasitoid seem to be belong to hydrolases classes, a property shared by venoms of numerous hymenopteran or non-hymenopteran species (see Table 1.2 at the end of paragraph) (Moreau and Guillot, 2005). In other organisms, venom hydrolases often have cytotoxic and histolytic effects once injected in hosts, preys or enemies (Schmidt *et al*., 1986). By their function of cell and/or tissue disruption, they can constitute self supporting factors or facilitate the spreading and action of other components (i.e. neurotoxic, paralytic, immune-suppressive, pain-inducing, anticlotting, antimicrobial or cytotoxic factors) (Bettini, 1978; Piek, 1986; Basu *et al*., 1990; Kuhn-Nentwig, 2003).

The venom of the ectoparasitoid *B. hebetor* contains toxins which block the neuromuscular transmissions in lepidopteran hosts by inhibiting the exocytosis of presynaptic vesicles (Piek, 1966; Walther and Reinecke, 1983). Several neurotoxins were isolated and partially or fully characterized: A-MTX and B-MTX (Visser *et al*., 1983), Brh-I, Brh-III and Brh-V (Quistad *et al*., 1994), BrhTX-1 and BrhTX-2 (Windass *et al*., 1996).

The composition in amino acids of A-MTX (43.7 kDa) and B-MTX (56.7 kDa) toxins has been determined but their sequences have not been fully investigated (Visser *et al*., 1983).

BrhTX-1 is a tetrameric protein composed of four polypeptides (BrhTX-1(a), BrhTX-1(b), BrhTX-1(c) and BrhTX-1(d)) having estimated molecular masses of 15, 17, 21 and 34 kDa, respectively (Windass *et al*., 1996). BrhTX-1 represented 7% of the starting paralysing activity present in extracts of venom glands and 0.5% of soluble venom gland proteins. Three out of the four subunits of the paralysing BrhTX-1 toxin share sequence identities either

with phospholipases A2 (BrhTX-1(b) and BrhTX-1(c)) or with a family of chymotrypsin-like proteases (BrhTX-1(d)) from various organisms (unpublished sources cited in Weaver *et al*., 2001). These putative enzymes could participate to the diffusion of neurotoxic venomous components in host tissues. The fourth subunit, BrhTX-1(a), shows sequence identities with mouse membrane-associated mucin muc3 (accession number AAH58768; 31% amino acid identity) and mouse hepatocyte growth factor (accession number NP_034557; 29% amino acid identity). In mouse, these proteins exert mitogenic, motogenic, morphogenic, and antiapoptotic activities for a wide variety of cells (Wang *et al*., 2002; Yoshida *et al*., 2003). The precise biological role of BrhTX-1(a) and the mechanism by wich BrhTX-1 subunits may cooperate to induce paralysis have yet to be fully resolved.

Windass *et al*. (1996) also claimed to have isolated another neurotoxic protein, termed BrhTX-2. The paralytic potential of BrhTX-2 has not been assessed in detail but its molecular structure is known: like BrhTX-1, BrhTX-2 is composed of four subunits of 17, 18.5, 21 and 34 kDa, respectively (Windass *et al*., 1996).

Brh-I, Brh-III and Brh-V (73 kDa) were sequenced and the two latter toxins were found to share similarities in their amino-terminal sequences (Quistad and Leisy, 1996) and seems to have high insecticidial potencies against *Heliotis* spp. and other lepidoptera larvae, with LD_{50} values ranging from 2 to 300 pmol/g. However, being large proteins (and fairly labile) they would be unsuitable for direct use as insecticides, although no dubt the possibility of including them in GM plants has not gone unconsidered (Quicke, 2014). Following a lag, new molecular tools are again making this an attractive choice of subject (Baker and Fabrik, 2000; Hartzer *et al*., 2005). Kryukova *et al*. (2011) showed that *Bracon* venom induces a general immune impairement in its laboratory host *G. mellonnella* and this is partly the result of suppression of the host's phenoloxidase system, which is involved in melanisation and

encapsulation. This reduction of the host's ability to heal the feeding site benefits the feeding of *Bracon* larvae. However, it also renders the host more susceptible to attack by other pathogens that may be present, such as entomopathogenic ascomycete fungi (Kryukov *et al*., 2013).

A recent study by Martinez *et al*. (2000) showed that the CfeHex2 hexamerin from the ant *Camponotus festinatus* possessed strong sequence identities (44.3%) with Brh-I. This finding did not clarify the potential mode of action of Brh-I since hexamerins constitute the main lipid-storing proteins of insect hemolymph and were not particularly known to induce neurotoxic effects.

Several other toxins were isolated from *B. hebetor*'s venom with molecular masses ranging from 16 to 30 kDa (Slavnova *et al*., 1987; Johnson *et al*., 1996; 1999). It remains to be determined if these proteins are related to those identified previously (Visser *et al*., 1983;, Quistad *et al*., 1994; Windass *et al.*, 1996).

Since the ability of parasitoids to control populations of insect pests represents their main applied value, the agronomic sector would naturally constitute the first field of application for parasitoid venom proteins.

Industrials have already manifested their interest towards these molecules as attested, for example, by several patent applications concerning *B. hebetor* venom neurotoxins (Quistad and Leisy, 1996; Windass *et al.,* 1996; Johnson *et al*., 1996; 1999). However, despite that they were reported to induce a diversified array of specific effects on the physiology of aphid, lepidopteran or dipteran pests, no parasitoid venom protein has yet been used as a pest control agent. This restriction may come from the fact that venom proteins would need to be injected into host hemolymph to induce their paralysing, immune suppressive, growth disruptive or castrating effects and would probably be prone to degradation in environment if delivered by insecticidal sprays (Moreau and Guillot, 2005). Such limitations could be partly removed by the use of fusion proteins expressed by transgenic plants to orally deliver

venom proteins to the hemolymph of insect pests. Recently, this approach has been successfully tested by fusing snowdroplect in to an insecticidal spider venom toxin (Fitches *et al*., 2001). Since the lectin resists gut proteolysis, binds to glycoproteins on the surface of the gut epithelium and can be detected in the hemolymph of orally exposed insects (Fitches *et al.,* 2004), it could constitute a promising system to target insecticidal proteins to internal tissues of pests.

More recently, Hardy *et al*. (2013) showed that it was possible to isolate spider-venom peptides with hight levels of oral insecticidial activity by directly screening for *per os* toxicity. An orally active insecticidal peptide (OAIP-1) from venom of the Australian tarantula *Selenotypus plumipes* was isolated and the oral LD_{50} determined in the agronomically important cotton bollworm *Helicoverpa armigera* was 104.2 ± 0.6 pmol/g; which is the highest *per os* activity reported to date for an insecticidal venom peptide.The three-dimensional structure of OAIP-1 determined using NMR spectroscopy revealed that the three disulfide bonds form an inhibitor cystine knot motif; this structural motif provides the peptide with a high level of biological stability that probably contributes to its oral activity. On a molar basis, OAIP-1 is more potent than any of the chemical pyrethroid insecticides. As showed by Hardy *et al*. (2013) the oral potency of OAIP-1, its rapid insecticidal action (i.e., death within 24-48 h), and its easy production via chemical or recombinant methods makes this peptide a good candidate for deployment as a foliar spray against lepidopterans and possibly other pest insect species. Moreover, the toxin should degrade in the environment to innocuous breakdown products. Since OAIP-1 is a genetically encoded peptide toxin it should also be possible to engineer transgenes encoding OAIP-1 into plants. OAIP-1 is likely to be synergized by the gut-lytic activity of the *Bacillus thuringiensis* Cry toxin (Bt) expressed in insect-resistant transgenic crops, and consequently it might be a good candidate for trait stacking with Bt.

Finally, the most innovative and promising potential of parasitoid venom proteins probably concerns biomedical applications. In fact, considerable number of peptides, proteins and enzymes remain to be described in parasitoid venoms and these components could represent an unsuspected source of new therapeutic agents. Several works have already opened the way in the use of insect peptides for biomedical purposes, demonstrating their effectiveness against viruses, pathogenic bacteria, and tumours (Slocinska *et al*., 2008; Chernysh *et al*., 2002; Tzou *et al*., 2002; Kuczer *et al*, 2008).

Table 1.2. Enzymatic activities and enzymes described in venom of parasitoid Hymenoptera.From Moreau and Guillot, 2005.

Enzyme names	**Enzymatic classes (EC)**	**Accession no. (Reference)**	**Methods of identification**	**Molecular masses (kDa)**
Laccase (putative)	1.10.3.2	CAD20461 (Parkinson *et al*., 2003)	FLS	76
Phenoloxidase I	1.14.18.1	CAC4150 (Parkinson *et al*., 2001)	FLS+EA+PS	79.5
Phenoloxidase II	1.14.18.1	CAC4149 (Parkinson *et al*., 2001)	FLS+EA+PS	79
Phenoloxidase III	1.14.18.1	CAC4148 (Parkinson *et al*., 2001)	FLS+EA+PS	79.3
Phospholipase A2 (putative)	3.1.1.4	CAB42199 (Windass *et al*., 1996)	FLS	21
Phospholipase A2 (putative)	3.1.1.4	CAA03259 (Windass *et al*., 1996)	FLS	17
Hyaluronoglucosaminidase	3.2.1.35	Doury *et al*., 1997	EA	n.d.
Chitinase	3.2.1.14	A53918 5 (Krishnan *et al*., 1994)	FLS+EA	52
Trehalase	3.2.1.28	CAD31109 (Parkinson *et al*., 2003)	FLS	61
Angiotensin-converting enzyme	3.4.15.1	Dani *et al*., 2003	EA+I	74
Serine protease (putative)	3.4.21.-	CAC42231(Parkinson *et al*., 2002)	FLS	30 and 28 (isoforms ?)
Endopeptidase	3.4.21.-	Dani *et al*., 2003	EA	n.d
Endopeptidase (putative)	3.4.21.-	CAB42201 (Windass *et al*., 1996)	FLS	34
Aminopeptidase	3.4.11.-	Dani *et al*., 2003	EA	n.d.
Metalloprotease (putative)	3.4.24.1	CAD21587 (Parkinson *et al*., 2002)	FLS	39.9
Aspartylglucosaminidase (putative)	3.5.1.26	Moreau *et al*., 2004	PS+I	30 and 18 (subunits)

Accession number. of enzymes at the Protein database of the NCBIEntrez retrieval system and/or reference of their first reports are given in the third column. EA, enzymatic assays; I, immunostaining; FLS, Full lengh sequencing; PS, partial sequencing.

1.7 Aims of the thesis

1.7.1 Overall project aim

The present project aims at identifying the role of the venom produced by *B. nigricans* ectoparasitoid wasps in the host regulation process, in order to identify bioactive components acting as candidate bioinsecticides, that can negatively interfere with host physiology and development.

1.7.2 Specific objectives

1. Host-regulation and major host alterations analysis (see Chapter 2).

This objective will be devoted to the analysis of the major host changes induced by *B. nigricans* parasitism. *S. littoralis* larvae exposed to parasitism will be studied in order to assess if and how their paralysis is associated with metabolic changes or alteration of their biochemical profile. Alterations in envenomated larvae, obtained by removing the parasitoid egg or with artificial injection of venom, will also be studied.

2. Identification of bioactive components of the venom (see Chapter 3).

The results obtained from objective 1 will be used to define specific assays to guide the purification process of the bioactive components of the venom that may play a role in the host regulation process. Venom proteins and peptides will be separated by electrophoretic and chromatographicmethods and fractions will be tested with the assays developed above. Injections of venom (crude and fractionated) will be performed and changes in the protein profile of the host hemolyph will be analyzed.

3. Transcriptomic and proteomic analyses of *B. nigricans* venom (see Chapter 3).

The analytical approach described in the objective 2 will be complemented by a transcriptomic and proteomic analysis of the venom gland of *B. nigricans*. RNAseq characterization of the gland transcriptome will be carried out and matched with the major protein components identified in the venom collected from the reservoir, by using SDS-PAGE electrophoresis and LC/MS-MS analysis.

4. Production of silver nanoparticles and action against *Hylastinus obscurus* (see Chapter 4).

The aim of this part of thesis conducted in Chile was to verify the entomocidal effects of silver nanoparticles against the red clover root borer, *Hylastinus obscurus* (Marsham) (Coleoptera: Curculionidae), a curculionid pest of *Trifolium pratense* L.

CHAPTER 2: Host-regulation and major host alterations analysis

2.1 Introduction and aim of the work

The host-regulation exerted by ectoparasitoids is mainly due to the injection of venom by wasp females into the hosts that induces rapid permanent paralysis, which allows the female oviposition and subsequently an undisturbed host feeding by the ectoparassitoid larvae.

Although more typical of endoparasitoid venom, in some cases also the venom from ectoparassitoids induces behavioral and metabolic transformation in the host and the impact of the venom is critical to the success of parasitism. Due to this evidence, the first part of the thesis concerns the analysis of the major changes induced by *B. nigricans* parasitism in our model host, the larvae of the Lepidoptera *S. littoralis*. Host larvae exposed to parasitism have been studied in order to assess if and how their paralysis is associated with metabolic changes and, in this case, characterize the alteration in their biochemical profile. Moreover, it has been assessed whether the cellular immune response, is altered, at different times after parasitization, because immune reactions may interfere with larval feeding. All biological observations has been also performed in envenomated larvae, obtained by removing the parasitoid egg or with artificial injection of venom.

2.2 Materials and methods

2.2.1 Experimental animals

The insects used for this work were obtained from a permanent rearing of *S. littoralis* (Boisduval) (Lepidoptera: Noctuidae) - *B.nigricans* Szépligeti

(Hymenoptera: Braconidae) at the laboratory of Entomology "E. Tremblay", Department of Agricolture, University of Naples "Federico II".

The adults of *B. nigricans* to start the rearing were gently provided by Prof. Zappalà Lucia from the University of Catania.

S. littoralis larvae were collectively reared from the first to the third larval stage on an artificial diet in plastic containers (25×10×7 cm) at 25 ± 1 °C and 70 ± 5% RH, with 16:8 h light-dark period; from the fourth larval stage, the larvae were individually reared on artificial diet in plastic jars (6 cm high × 2 cm of diameter) closed with cotton wool at the same conditions. The artificial diet has the following composition: 41.4 g/L wheat germ, 59.2 g/L brewer's yeast, 165 g/L corn meal, 5.9 g/L ascorbic acid, 1.53 g/L benzoic acid, 1.8 g/L methy4-hydroxybenzoate and 29.6 g/L agar. The different larval stages were identified on the basis of the morphological characteristics described by Mochida (1973).

Adults of *B. nigricans* were reared in Petri dishes (6 cm in diameter) containing a *S. littoralis* larvae at the first day of the fifth instar and fed with honey drops, accordingly to Zappalà *et al*. (2012), with minor changes. The ectoparasitoids were maintained at 27 ± 1 °C, relative humidity of 70 ± 5% and 16:8 h light-dark period until the parasitization. After 1 day the adults of ectoparasitoids were transferred in a new Petri dish containing another *S. littoralis* fifth instar larvae.

Parasitized *S. littoralis* larvae were then kept in a climatic chamber (25 ± 2 °C, HR 70 ± 5%, 18L: 6D) for the duration of the development cycle of the parasitoid, which lasts about 12-13 days. In these conditions, 48 h after the oviposition on the host body the eggs hatch and the newly emerged larvae begin to feed on the body fluids. After about 3 days begins the pupal stage, and after 7 days of incubation emerge new adults.

The biological material for the experiments consists of *S. littoralis* fifth instar larvae (first day) aged and adults of *B.nigricans* only fed with honey for at

least 24 hours before the start of the bioassay. This protocol appears to speed up the time of oviposition once the parasitoid comes in contact with the host.

2.2.2 Number and viability of *S. littoralis* hemocytes

Total hemocytes counts and viability tests were performed on larvae to which the following treatments were applied:

- natural parasitism by the parasitoid (parasitized larvae);
- natural parasitism by the parasitoid with subsequent removal of the eggs laid by using a fine-tipped brush dipped in water (paralyzed larvae);
- no parasitism (healthy larvae, control).

The parasitism was carried out by exposing single *S. littoralis* fifth instar larvae (first day), to *B. nigricans* adults (2 females and 2 males) in a Petri dish (6 cm in diameter) used as an arena. The larvae used as a control were placed in Petri dishes (6 cm in diameter) with a piece of artificial diet until use.The larvae were placed in cell conditioned at 25 °C ± 2 °C, 30% RH, with a photoperiod of 16L:8D. To optimize the parasitism, before testing, the females of the parasitoid were kept for two days in Petri dishes only with honey.The bioassay was carried out at different times (0, 3, 6, 12, 24 hours) from the oviposition of the eggs. Time zero corresponds to the completion of envenomation and oviposition. For each time and treatment 6 larvae were tested.

Treated larvae, placed individually in plastic jars, were anesthetized on ice for a few minutes, sterilized in 70% ethanol and rinsed in double distilled water (ddH_2O). After being dried, larvae were placed on parafilm and cut at the level of the third pair of thoracic legs with micro dissecting scissors, properly sterilized in 100% ethanol. A slight pressure on the abdomen allowed the hemolymph flow. Hemolymph was thus taken by a micropipette (20 µl of hemolymph/larva) and collected in an eppendorf tube containing 100 µl of

MEAD anti-coagulant buffer (98 mM NaOH, 145 mM NaCl, 17 mM EDTA, 41 mM citric acid, pH 4.5), kept on ice.

Hemocytes were separated from plasma by 10 min centrifugation at 300 × *g*, at 4 °C. The pellet containing the hemocytes was resuspended in 10 µl of cold PBS (Phosphate Buffered Saline) and kept on ice. 9 µl of the suspension were transferred into a new eppendorf containing 1µl of trypan blue dye (0.4% w/v).

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue (Strober, 2001) while the dye enters into dead cells conferring them a different colour. After gentle pipetting, the suspension was loaded into a Bürker chamber. The counting of hemocytes was carried out with the optical microscope Zeiss Axioskop with a 40X objective. The total hemocytes number (cells/ml) and cell viability (dead cells/ml) were calculated with the following formula:

$$N°cell/ml = \frac{N}{24} \times FD \times 250 \times 10^3$$

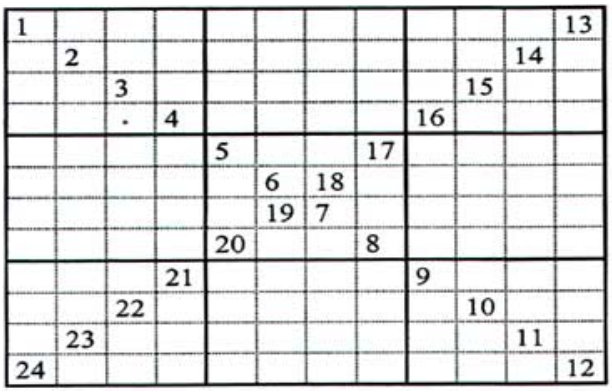
where:

N= ∑ of counted hemocytes inside the mean squares along the diagonals of each cell in the Bürker chamber.

FD = dilution factor corresponding to $\frac{\text{final volume loaded}}{\text{initial volume collected}} = \frac{10\mu l}{9\mu l} = 1.1\mu l$

250= factor that considers the volume of each mean square Bürker chamber (1/250 mm^3).

10^3= conversion factor from µl to ml 24= number of mean squares which form the diagonals of the cell according to the following scheme:



2.3 Metabolic and biochemical regulation of *S. littoralis*

The metabolic and biochemical alterations that develop during parasitization are different and complex, ranging from changes in nutrient profiles to the regulation of energy balance of the host (Alleyne *et al*., 1997; Sak *et al*., 2011; Beckage and Gelman , 2004; Thompson *et al*., 2005). The objective of the following experiments is to describe the impact of the parasitization on the metabolism of *S. littoralis* larvae by measuring the CO_2 produced during the first days of development of the parasitoid and the changes in the nutrient composition of host hemolymph in terms of proteins and carbohydrates.

2.3.1 Quantification of CO_2 emission

The measurement of CO_2 dispersed has been performed on parasitized larvae, natural envenomated larvae (parasitized larvae from which the eggs have been removed) and healthy larvae as a control. The parasitization was carried out as described in paragraph 2.2.2.

After oviposition, three experimental groups were prepared:

- parasitized larvae;
- parasitized larvae deprived of the eggs (envenomated larvae);
- non parasitized larvae (control).

The measurement of CO_2 dispersed was performed by incubating groups of 5 larvae in controlled conditions of temperature, humidity and photoperiod (25

°C, 50% RH, 16L:8D). All incubations were carried out in glass hermetic containers, placing 5 larvae individually isolated in plastic jars, a vial with 25 ml of water and another vial with 10 ml of 0.5 M NaOH (see Figure 2.1). Soda captures the CO_2 from larvae respiration forming Na_2CO_3.

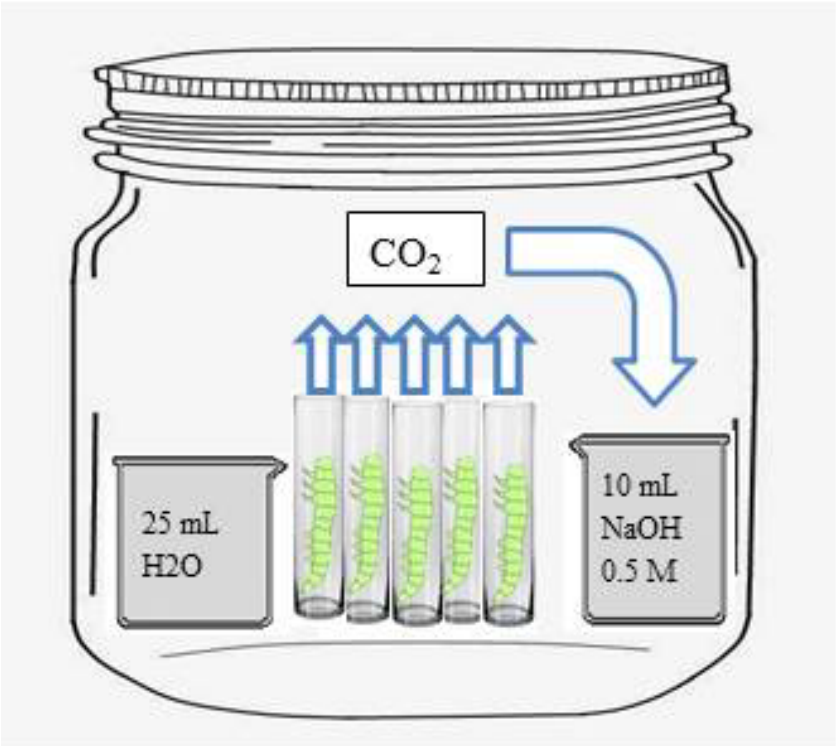


Figure 2.1. Schematic drawing of samples's incubation.

In addition, a blank incubation was carried out: a jar with soda and water without *S. littoralis* larvae.

At the end of the incubations, the jars containing the NaOH has been collected and added with 5 ml of 20% $BaCl_2$ which facilitate the precipitation of the carbonate along with a few drops of phenolphthalein, the acid-base indicator chosen for the titration. This molecule changes colour from pink to colourless when pH decreases to pH<8. The titration of the formed Na_2CO_3 was then

carried out with HCl 0.5 M. A control titration by titrating 10 ml of NaOH with known concentration (0.5 M) has been also performed.

CO_2 measurements (mg/larva) were performed at 24, 48 and 72 h of incubation. At each sampling time, the jar with NaOH from the glass container was replaced immediately with another bottle containing 10 ml of NaOH.

The determination of the CO_2 dispersed by the individual larva was measured according to the following calculation:

$$\text{mg } CO_2 \text{ / larva} = (B\text{-}C) \times M \times 22 \times (1/5) \text{ / h spent}$$

where:

B = value of the volume of the solution of HCl used for the blank.

C = value of the volume of HCl solution used for the sample.

M = molarity of HCl solution used to titrate the NaOH at known concentration.

22 = equivalent weight of C in CO_2.

1/5 = factor to calculate the value for each tested larvae.

For each treatment and each time 3 replicates were performed.

2.3.2 Measurement of protein concentration in *S. littoralis* hemolymph

The quantification of proteins in the hemolymph of *S. littoralis* larvae was performed as described by Bradford (1976) using the reagent Dye Reagent Concentrate (Biorad) using bovine serum albumin as standard.

The assay was carried out on the hemolymph collected from the following samples:

- parasitized larvae;
- artificially injected larvae. Venom reservoir content from *B. nigricans* females was extracted in 20 µl PBS, kept on ice. Following centrifugation

(5000 × *g* for 5 min at 4 °C) to remove cell debris, final venom concentration was adjusted to 0,1 venom reservoir equivalent (VRE) or female equivalent (FE). A 5 µl solution of venom preparation (0,1 FE) was injected in the neck membrane of *S. littoralis* fifth instar larvae (first day), using a 25 µl Hamilton Microsyringe (Hamilton,USA);

- untreated larvae (controls).

Hemolymph collection was performed at 0-, 12-, 24- and 48- post treatments (parasitization or venom injection). Larval hemolymph was collected from a cut of the abdominal proleg into an Eppendorf tube containing a few grains of N-phenylthiourea to prevent melanization. 30 µl of hemolypmh from each individual larva were collected at each time period and for each treatment.

The hemolypmh was spun at 1600 × *g* for 10 min at 4 °C to remove hemocytes. The supernatant (plasma) was transferred to a new Eppendor tube. Five microliters of plasma were used for protein quantification and the remaining volume was stored at -80 °C.

The plasma was diluited 1:20 with ddH_2O. Total protein concentration was measured according to Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard in a Biophotometer Eppendorf at 595 nm. Proteins determinations were repeated 6 times for each experimental group.

2.3.3 Polyacrilammide gel electrophoresis of *S. littoralis* hemolymph

Significant quantitative changes of hemolymph proteins was registered in *S. littoralis* parasitized by *B. nigricans*. The present experiment was designed to assess qualitative changes of hemolymph proteins to complete the overview on these biochemical changes. It was also assessed any possible role of progeny salivary secretions and venom proteins, injected artificially and naturally by parasitoids, in determining the major protein changes in the larval hemolymph at 48 h post treatments.

The bioassay was carried out by subjecting the larvae to the following treatments:

- natural parasitization by the parasitoid was carried out by placing in a Petri dish each larva of *S. littoralis* fifth instar larvae (first day) with 2 females and 2 males of *B. nigricans* at 25 °C until the oviposition;
- natural parasitization by the parasitoid *B. nigricans* with subsequent removal of the eggs laid by using a fine-tipped brush dipped in water;
- artificial injection of venom. Venom reservoir content from a *B. nigricans* female was extracted in 20 µl of cold PBS. Following centrifugation (5000 × g for 5 min at 4 °C) to remove cell debris, final venom concentration was adjusted to 0.1 venom reservoir equivalent (VRE) or female equivalent (FE). A 5 µl solution of venom preparation (0.1 FE) was injected in neck membrane of *S. littoralis* fifth instar larvae (first day), using a 25 µl Hamilton Microsyringe (Hamilton, USA).

Controls consisted of unparasitized larvae and injected with 5 µl of PBS.

Hemolymph was collected at 48 h post each treatments, by a cut of the abdominal proleg and transferred into an Eppendorf tube; then, it was centrifuged at 1000 × g for 2 min at 4 °C to remove hemocytes.

The supernatant (plasma) was transferred into a clean Eppendorf tube.

A sample of plasma (13 µg of proteins) was loaded into a 4-12% gradient separating gel and run under 200 V and costant current conditions.

The gel was fixed (30% ethanol, 10% acid acetic), stained for 45 min in 0.2 % (w/v) of silver nitrate solution and developed in a solution containing 3 % (w/v) sodium carbonate, 0.025 % (v/v) formaldehyde and 10 mg/ml sodium thiosulfate.

2.3.4 Quantification of total carbohydratesin *S. littoralis* hemolymph

The titer of carbohydrates in hemolymph was estimated by the anthrone method, which is a simple colorimetric method with relative insensitivity to

interferences from other cellular components. The first step in total carbohydrates measurement is to hydrolyze the polysaccharides and to dehydrate the monomers. The 5-carbon (pentoses) and 6-carbon (hexoses) sugars are converted to furfural and hydroxymethylfurfural, respectively. When anthrone reacts with these digestion products it gives a colored compound. The amount of total carbohydrates in the sample was estimated reading the adsorbance of the resulting solution against a glucose standard curve. The quantification of total carbohydrates circulating in the hemolymph of *S. littoralis* larvae was performed as described by Roe (1955) and Salvador and Consoli (2007) using the anthrone reagent (Sigma) with some modifications.

The bioassay was carried out on the following samples:

- parasitized larvae;
- artificially injected larvae;
- untreated larvae (controls).

Hemolymph collection was performed at 0-, 12-, 24- and 48- post treatments (parasitization or venom injection).

Larval hemolymph (30 µl) was collected from a cut of the abdominal proleg into an Eppendorf tube containing the same quantity (30 µl) of MEAD anti-coagulant buffer. The hemolypmh was spun at 1000 × g for 2 min at 4 °C to remove hemocytes. The supernatant (plasma) was transferred to a clean Eppendor tube.

Ten microliters oh hemolymph suspension was used for total carbohydrates analysis and the remain samples was kept at -80 °C.

800 µl of anthrone reagent (freshly prepared 0.2 % w/v in 96% H_2SO_4) were added to each sample of plasma (400 µl of plasma diluted 1:40 with ddH_2O) and the mixtures were placed at 100 ° C for 5 min in a thermomixer. 100 µl of

each incubation mixture were transferred into a 96 well-plate and the absorbance was read at 620 nm using a Multiskan Ex reader (Thermo).

The calibration curve was prepared with the same protocol, by using a standard solutions of D-Glucose (0, 20, 40, 60, 80, and 100 mg/L). For both standards and samples the analysis was carried out in triplicate by averaging the values obtained.

2.3.5 Cellular modifications

S. littoralis fifth instar larvae unparasitized and 48 h parasitized as described above, were fixed in 2% glutaraldehyde (in 0.1M Na-cacodylate buffer, pH 7.2), for 2 h at room temperature. Specimens were then post-fixed for 2 h with 1% osmic acid in 0.1 M Na-cacodylate buffer at room temperature, and dehydrated in an ethanol series.

For transmission electron microscopy (TEM) preparation, dehydrated samples were embedded in an Epon-Araldite 812 mixture and sections were cut with a Reichert Ultracut S ultratome (Leica, Nussolch, Germany) according with Grimaldi *et al*., 2006. Semithin sections were stained by conventional methods (crystalviolet and basic fuchsin) and observed with a lightmicroscope (Olympus, Tokyo, Japan). Images were acquired with a Leica DC 300F camera.

Thin sections were stained with uranyl acetate and lead citrate, and observed with a Jeol 1010 EX electronmicroscope (Jeol, Tokyo, Japan).

2.4 Statistical analysis

Means were compared using one- or two-way analysis of variance (ANOVA) and subsequently, means were separated using LSD's post-hoc test. SPSS (version 18.0 for Windows, SPSS Science, USA) was used for data analysis. Results were considered statistically significant when $P<0.05$.

2.5 Results

2.5.1 Effect of parasitism on hemocytes

Parasitized host larvae have a similar hemocyte number compared to unparasitized controls, but, by 24 hours after parasitism, they failed to show the increase of circulating cells registered in healthy controls. In fact, at 24 h after treatments, the hemocyte number was significantly higher in controls, compared to parasitized and envenomated larvae (one-way ANOVA, $P<0.001$) (Figure 2.2).

Hemocytes viability evidenced by trypan blue staining shows identical cell death at all time points sampled and between treatments. In fact there are no significant differences between parasitized, envenomated and unparasitized larvae and the percentage of mortality is 5% (Figure 2.3).

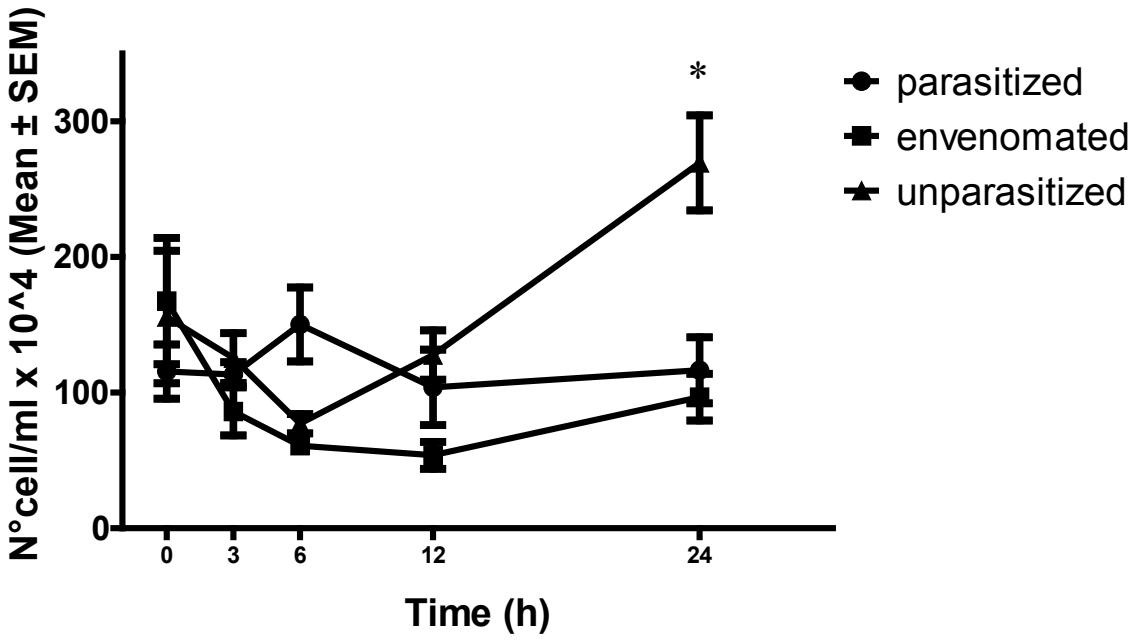


Figure 2.2. Total hemocyte counts from fifth instar larvae of *S. littoralis* as affected by *B. nigricans* parasitism and natural envenomation, obtained by parasitoid egg removal (*$P<0.001$, one-way ANOVA, LSD's post-hoc).

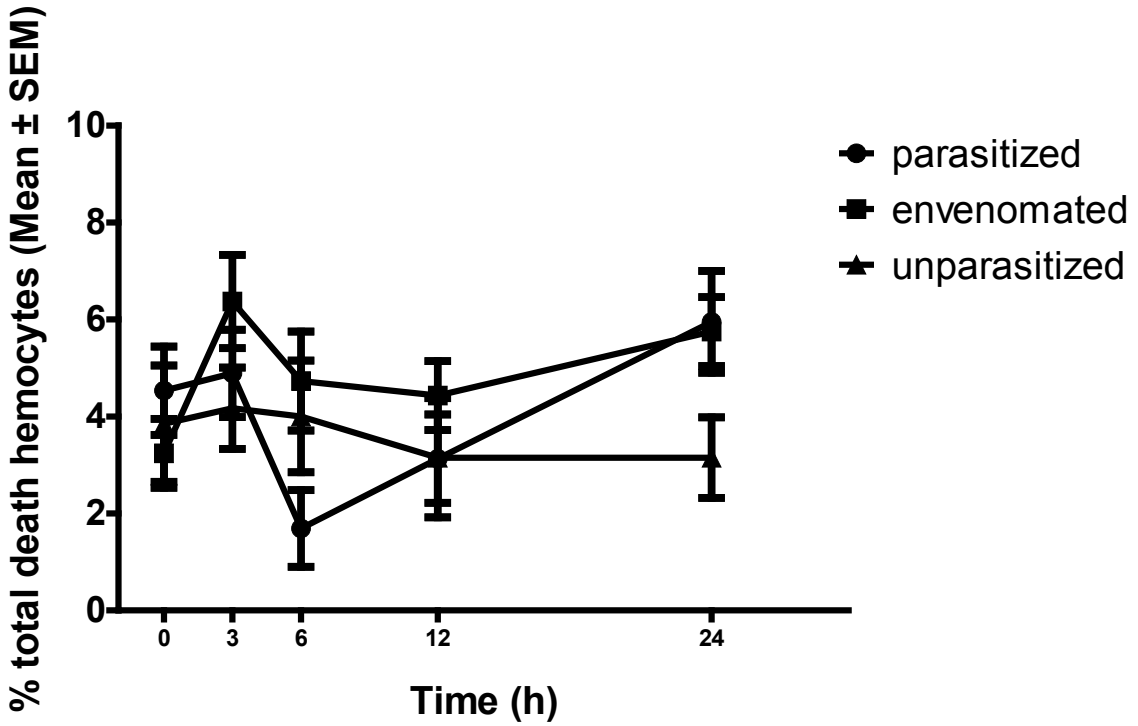


Figure 2.3. Percentage of dead hemocytes in parasitized, natural envenomated and unparasitized larvae, observed at 0, 3, 6, 12, and 24 hours after parasitization.

2.5.2 CO_2 emission rate and biochemical regulation induced by parasitism

In order to clarify the profile of host physiological redirection by parasitism, measurements of CO_2 produced by parasitized and non parasitized larvae were carried out in order to analyze wether parasitism affects host metabolic activity. The amount of CO_2 produced in parasitized larvae shows a significant reduction of host metabolic activity, and this physiological change was reproduced, along with paralysis, by host envenomation (one-way ANOVA, $P<0.01$) (Figure 2.4).

The amount of CO_2 produced by *S. littoralis* larvae showed a 50% reduction soon after parasitism, which persisted over the following 72 hours.

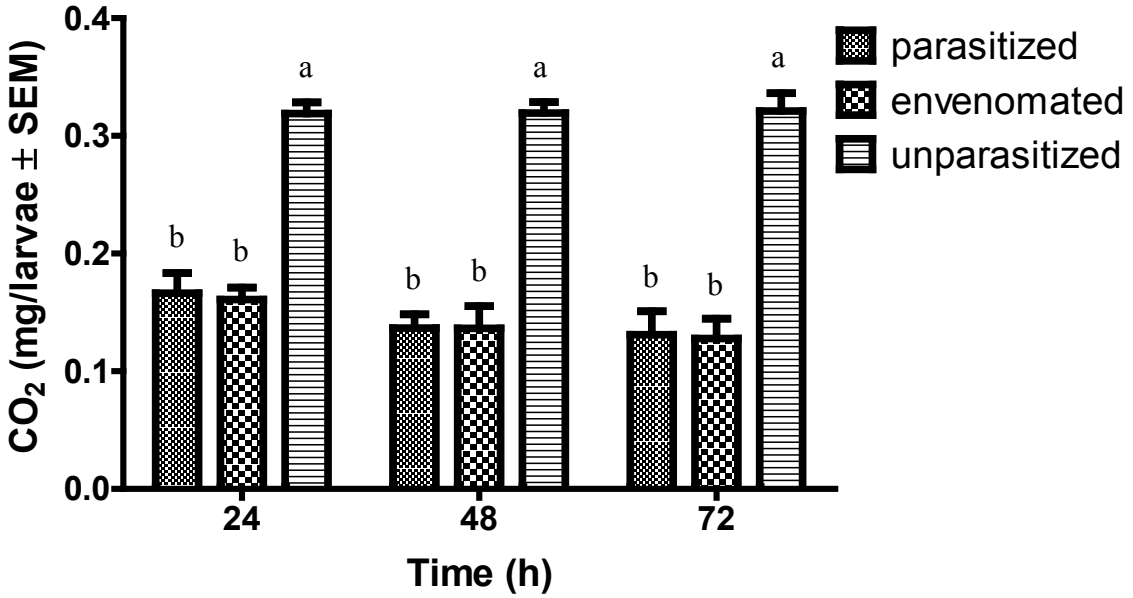


Figure 2.4. Emission of CO_2 (mg/larvae) by parasitized, naturally envenomated and non parasitized larvae at 24, 48, 72 hours after treatments (one-way ANOVA, LSD's post-hoc analysis for each time, different letters show statistically significant differences $P<0.01$).

Rapid paralysis of host larvae was followed by a change of the nutritional suitability of their hemolymph, which showed, compared to unparasitized larvae, a significant increased titer of proteins, at 48 h post parasitization, which was only partially reproduced by envenomation (one-way ANOVA, $P<0.05$) (Figure 2.5).

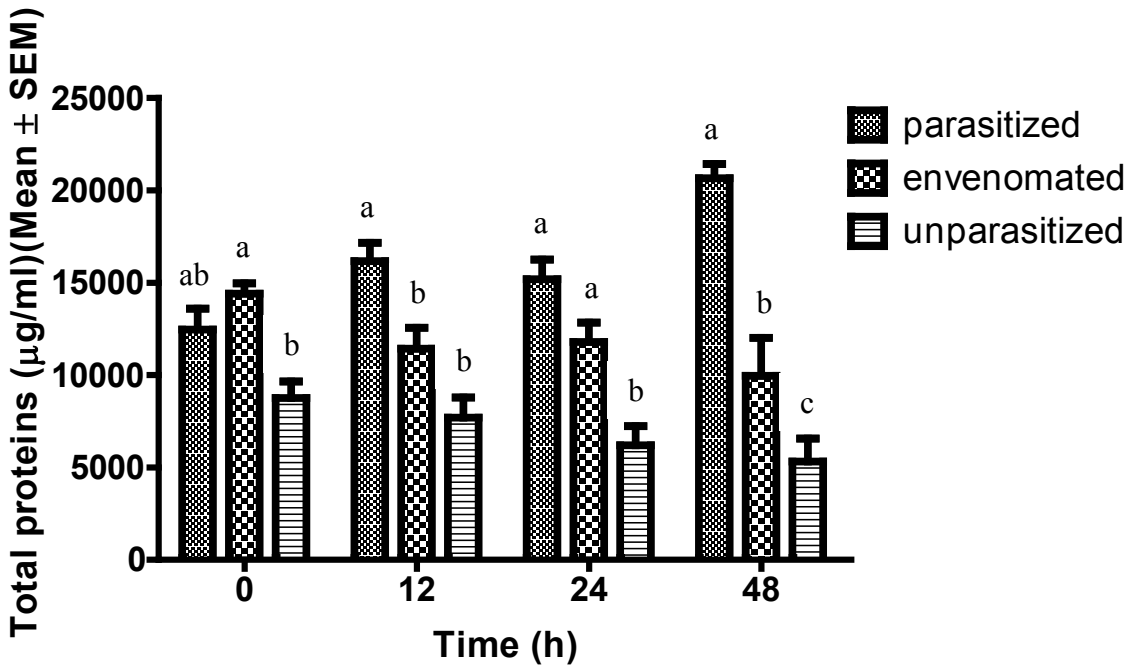


Figure 2.5. Total protein quantification in the hemolymph plasma of *S.littorali*s larvae as affected by parasitism and artificial envenomation by injection (one-way ANOVA, LSD's post-hoc analysis for each time, different letters show statistically significant differences $P<0.05$).

The ectoparasitoid *B. nigricans* altered the biochemical composition of the hemolymph in terms of free sugars circulating at 24 h post parasitization.

In fact, at 0, 12 and 48 h post treatments there was no statistically significant differences between parasitized, artificially envenomated and unparasitized larvae. At 24 h the level of sugars in hemolymph of parasitized larvae was significally higher in respect to the other groups; there was an evident difference also between envenomated and control larvae (one-way ANOVA, $P<0.05$) (Figure 2.6).

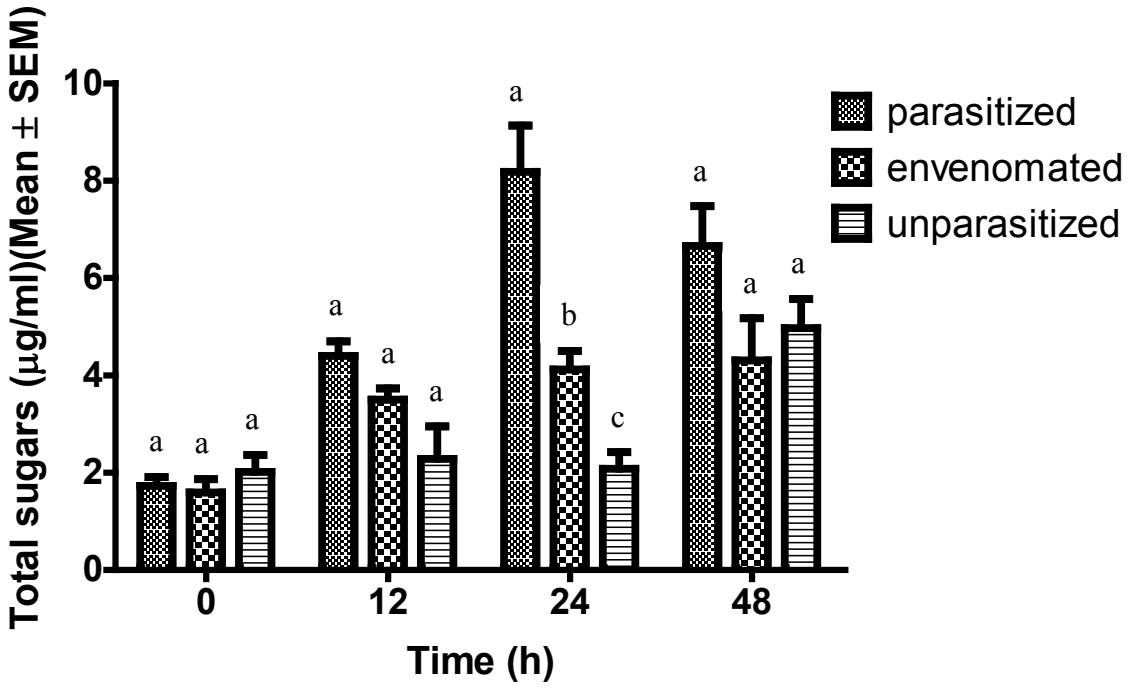


Figure 2.6. Total carbohydrates quantification in the hemolymph plasma of *S. littoralis* larvae as affected by parasitism and artificial envenomation by injection (one-way ANOVA, Games-Howell post-hoc analysis for each time, different letters show statistically significant differences $P<0.05$).

2.5.3 Proteic electrophoresis pattern of *S. littoralis* hemolymph

The electrophoresis profile of *S. littoralis* hemolymph shows difference in components between treatments (Figure 2.7).

In the hemolymph of paralyzed (P1) and parasitized (P2) larvae appeared various minor components and some relatively more abundant proteins or peptides. The hemolymph of healthy (C1) and PBS injected (C2) larvae shows the same protein profiles, with the only difference that in PBS injected larvae two or more bands appeared enlarged.

At last the hemolymph of larvae artificially injected (V) shows an intermediate profile between C and P group; in fact it presented bands with low molecular mass like in P1 and P2 and bands with high molecular mass like in C1 and C2. The peculiarity of V pattern was the evident presence of a protein band of 15 kDa.

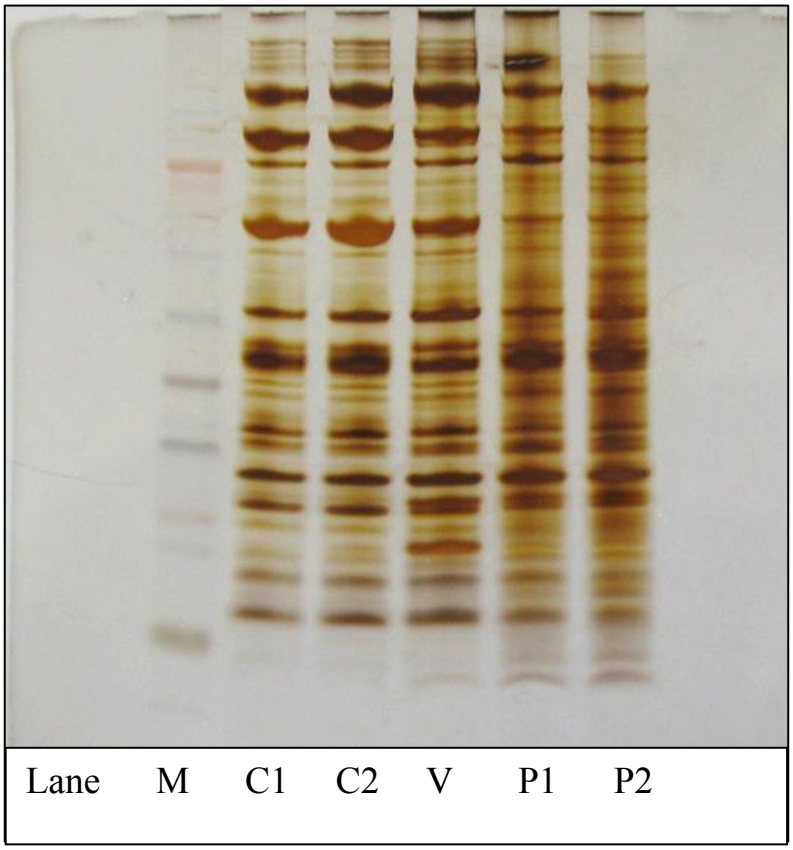


Figure 2.7. SDS-Polyacrylamide gel electrophoresis of hemolymphatic proteins of *S. littoralis* fifth instar larvae. (C1) Healthy, PBS injected (C2) 0.1 RE venom injected (V), naturally envenomated (P1) and parasitized (P2) larvae. Hemolymph samples were collected 48 h after treatments.

2.5.4 Modification of fatbody cells and hemocytes

Metabolic and biochemical alterations are associated with changes of both hemocytes and fat body that likely contribute to the mobilization of nutritional resources in the hemolymph to support parasitoid development. The resulting enhancement of host nutritional suitability is associated with structural changes of the fat body cells, which interact with hemocytes and show signs of cell degeneration (Figure 2.8).

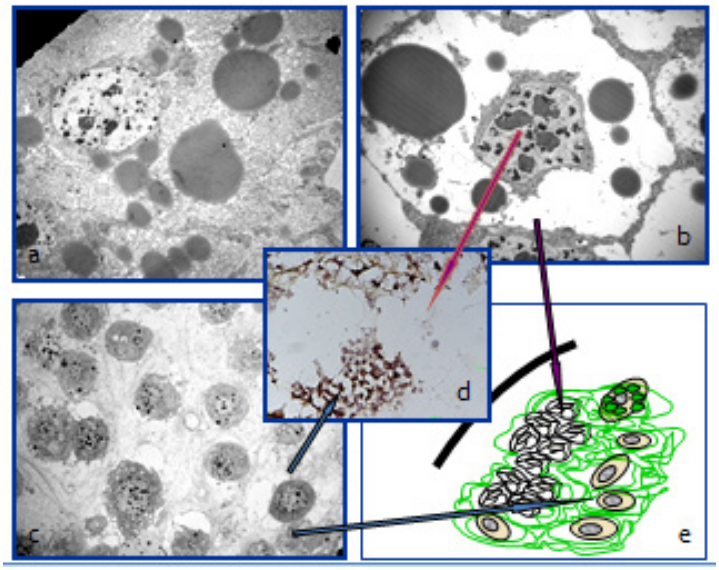


Figure 2.8 Fat body cells in unparasitized larvae show a dense cytoplasm (a), which is largely lost following parasitism (b), when cells are entrapped in a fibrillar matrix produced by hemocytes (c); broad view of interacting cells at low magnification (d) and represented in a schematic drawing (e).

2.6 Discussion and conclusion

Parasitism and venom related changes in hemolymph protein profiles and total protein levels have been detected in numerous parasitoid-host model systems (Sak *et al*., 2011). Not surprisingly, the impact of the parasitoids on the host hemolymph has been variable, and in many cases, the hemolymph protein changes seem to be part of the host conditioning necessary for the parasitoid's larvae to successfully complete development (Bae and Kim, 2004; Nakamatsu and Tanaka, 2003; Nakamatsu *et al*., 2001; Bischof and Ortel, 1996).

Altering the host nutritional condition for the benefit of wasp offspring is generally thought to be most common for koinobionts, and would presumably not be expected for an idiobiont like *B. nigricans*. Previous studies reported that the total quantity of hemolymph protein is reduced during ectoparasitism (Coudron et al., 1997; Altunas *et al*., 2010) and endoparasitism (Beckage and Kanost, 1993). Baker and Fabrick (2000) reported that parasitization of

Plodia interpunctella last instar larvae by *Habrobracon hebetor* did not qualitatively change plasma proteins when analyzed by SDS-PAGE, indicating that parasitization did not induce the appearance of novel proteins in host hemolymph.

The results of the present study, on the contrary, show an increment of protein concentration in the hemolymph of parasitized and envenomated larvae (the last with minor impact) 48 h post natural and artificial venom injection and when progeny starts to feed on body host fluids (Figure 2.5). These increments, according with the protein profile of hemolymph, suggest the hypothesis that the only venom, and not the saliva of offspring's larvae, stimulate the hydrolysis of hemolymph host nutrients, producing smaller and more suitable proteins, because P1 and P2 sample profiles (without and with offspring's salival enzymes action respectively) are identical (Figure 2.7). The increase in other proteins could be related to decomposition of the fat body and tissue proteins induced by parasitoid venom for host regulation. The hydrolysis of hemolymph proteins in minor components does not occur for healthy and PBS injected larvae where it was found the presence of proteins with hight molecular mass. Finally, the artificial injection of venom induces a minor increment of protein concentration at 48 h according with the hemolimphatic proteins profile that shows a limited degradation of proteins. A protein of 15 kDa in the hemolymph of venom injected larvae seems enlarged in respect to other treatments, confirming that the artificial injection induces different responces and partially reproduces a natural parasitism condition.

The effects of parasitization by hymenopteran species on the carbohydrate metabolism (in particular trehalose and glycogen level) in their lepidopteran hosts have been investigated in many studies, revealing incongruent effects (Bischof and Ortel, 1996). In *Manduca sexta* larvae parasitized by *Apanteles congregates* the hemolymph sugar concentrations were found to be reduced

(Dahlman, 1975; Thompson and Lee, 1993), whereas in *Heliothis virescens* parasitized by *Campoletis sonorensis* an opposite effect occurs since an increase of trehalose concentration was observed (Dahlman and Vinson, 1976). Contradictory effects were even reported for the same lepidopteran host species (*H. virescens*) when the latter was parasitized by two different species of braconid wasps (Dahlman and Vinson, 1975); trehalose levels in host hemolymph were not affected by parasitism by *Cardiochiles nigriceps* but nearly tripled after parasitization by *Microplitis croceipes*. Thompson (1986) also reported increases of hemolymph trehalose levels for *Trichoplusia ni* parasitized by *Hyposoter exiguae*, concluding that the parasitoids caused stress and induced hyperglycemia in the host. Parasitism of *Lymantria dispar* by the ectoparasitoid *Glyptapanteles liparidis*, on the other hand, led to increased hemolymph trehalose levels during the early endoparasitic phase but to a significant decrease at the end of its larval development; no effect of parasitism on the glycogen content was ascertained (Hoch *et al*., 2002).

In this study carbohydrates level increased just at 24 h post parasitization and that the artificial envenomation determines a minor increment but significant in respect to controls. At 48 h carbohydrates concentration in parasitized larvae decreased at control level, while in envenomated larvae the level remains the same (Figure 2.6). This difference suggest that likely the early feeding activity of progeny determines carbohydrates consumption, easily and readily assimilated nutrients. These results agree with the study on *B. hebetor* development conducted by Saadat *et al*. (2014) in which it is concluded that stored product insects, which normally feed on a diet rich in sugar and glycogen, provide physiological conditions that are more suitable for the parasitoid than field crop insects, which feed on diet rich in terpenes and tannins.

In the *B. nigricans* - *S. littoralis* system parasitism induces a growth arrest with a reduction of basal metabolism, in terms of CO_2 emission (Figure 2.4).

The 50% decline of CO_2 production is coherent with the lack of locomotor activity and feeding. The same decrease of respiratory metabolism was observed by River and Denlinger (1994) in the flesh fly, *Sarcophaga bullata*, envenomated by the pupal ectoparasitoid, *Nasonia vitripennis*.

Several authors have reported on the effects of parasitism on total and differential hemocyte counts in different insect hosts.

Recently, studies have been conducted to examine the effects of parasitism and venom from *P. turionellae* on hemocyte numbers in two developmental stages of the host, *G. mellonella* (Lepidoptera: Pyralidae) (Er *et al*., 2010). Total hemocyte count indicated a considerable decline in the number of circulating hemocytes in *G. mellonella* pupae and larvae exposed to *P. turionellae* or any dose of wasp venom below the lethal dose, 99% response (LD99) calculated for *G. mellonell*a pupae and larvae (Ergin *et al*., 2005) injected experimentally (Er *et al*., 2010). In several lepidopteran hosts, successful parasitization by parasitic wasps leads to a reduction in the total number of circulating hemocytes (Mochiah *et al*., 2003; Ibrahim and Kim, 2006). Decreases in hemocyte numbers and increases in hemocyte damage also occurred in experimentally envenomated insects (Richards and Edwards, 1999; Zhang *et al*., 2005; Yu *et al*., 2007). According with these previous studies, *B. nigricans* venom probably blocks the formation of new hemocytes, that results in a decrease in hemocytes number respect to the unparasitized larvae. In certain host parasitoid systems, the reduction of hemocytes in circulation was caused by their death (Rivers *et al*., 2010; Nakamatsu and Tanaka, 2003; Er *et al*., 2011; Teramoto and Tanaka, 2004; Richards and Dani, 2007; Rivers *et al*., 2007). Apoptosis and/or oncosis of hemocytes appear to be necessary means to manipulate the host to ensure successful development of parasitoid larvae (Rivers *et al*., 2010; Nakamatsu and Tanaka, 2003). Recently, studies have been conducted to examine the ability of *P.*

turionellae venom to induce cell death in the circulating hemocytes of the natural host *G. mellonella* at the larval and pupal stage (Er *et al*., 2011).

B. nigricans venom to the contrary, does not affects the viability of hemocytes, which appeared alive and with the same low percentage of mortality present in control larvae (Figure 2.3).

It is worth to note that at 48 hours after parasitization and natural envenomation, hemocytes were strongly aggregated and thus it was difficult to count them. Moreover, they appeared quite enlarged, with many granular inclusions, resembling the macrogranulocytes described by Zhai and Zhao (2012), actively involved in fat body remodeling at metamorphosis and that in this case was observed at 48 h after parasitization. Ribeiro and Brehélin (2010) show that this large cells filled with numerous dense granules, different from both granular hemocytes and spherule cells, are observed in *S. littoralis* but not in *S. frugiperda*. Larger, granulocyte-like hemocytes called hyperphagocytic cells, due to their extreme fagocytic activity, that differentiate following immune challenge, have also been described from the hawkmoth, *M. sexta* (Dean *et al*., 2004).

The TEM analysis showed the presence of macrogranulocytes, which have been reported as major players in fat body degradation during metamorphosis, by actively secreting cathepsin L (Wang *et al*., 2010; Zhai and Zhao, 2012; Yang *et al*., 2007) and showed structural changes of fat body tissue, which appeared rich in vacuoles and entrapped in a fibrillar matrix probably secreted by hemocytes and which supposed to be amyloids (Figure 2.8). As shows by Falabella *et al*. (2012) a protein produced by hemocytes of another noctuidae, *H. virescens* larvae, generates amyloid fibrils, which accumulate in large cisternae of the rough endoplasmic reticulum and are released upon immune challenge, to form a layer coating non-self objects entering the hemocoel. A study with specific markers is required to confirm the nature of this fibrillar matrix.

CHAPTER 3: Transcriptomic and proteomic analysis of venom apparatus

3.1 Introduction and aim of the work

Parasitoid wasps (Hymenoptera: Apocrita) constitute by far the largest group of parasitic insects with an estimated total number of approximately 250,000 species (Whitfield, 2013). As described in chapter 1, some species develop outside (ectoparasitoids) and others inside (endoparasitoids) the body of an insect or other arthropod host and, depending on the species, various stages of the host can be parasitized (egg, egg-larval, larval, pupal and adult-parasitoids). In ectoparasitoid species, venoms often induce paralysis and/or regulate host development, metabolism and immune responses (Doury *et al*.,1997; Abt and Rivers, 2007). A methodology increasingly attractive for the study of the composition ofvenoms is the combination of transcriptomic and proteomic analysis. The initial product of genome expression is the transcriptome, a collection of RNA molecules derived from those protein coding genes whose biological information is required by the cell at a particular time (Figure 3.1). These RNA molecules directs synthesis of the final product of genome expression, the proteome, the cell's repertoire of proteins, which specifies the nature of the biochemical reactions that the cell is able to carry out (Brown, 2002).

Broader studies have also previously investigated the composition of parasitoid venoms by the separate use of proteomic andtranscriptomic approaches combined with bioinformatic analyses (Vincent *et al*., 2010).

The aim of this part of the thesis is to have a comprehensive picture of the molecular profile of the venom gland secretome, by a transcriptome analysis.

The RNA-Seq characterization of the gland transcriptome was carried out and the sequences matched with the major protein components identified in the

venom collected from the reservoir, by using Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and Liquid chromatography-tandem mass spectrometry (LC/MS-MS) analysis. Finally, it provides a basic overview of the functional role played by the bioactive components isolated from *B. nigricans* venom in the host regulation of *S. littoralis*.

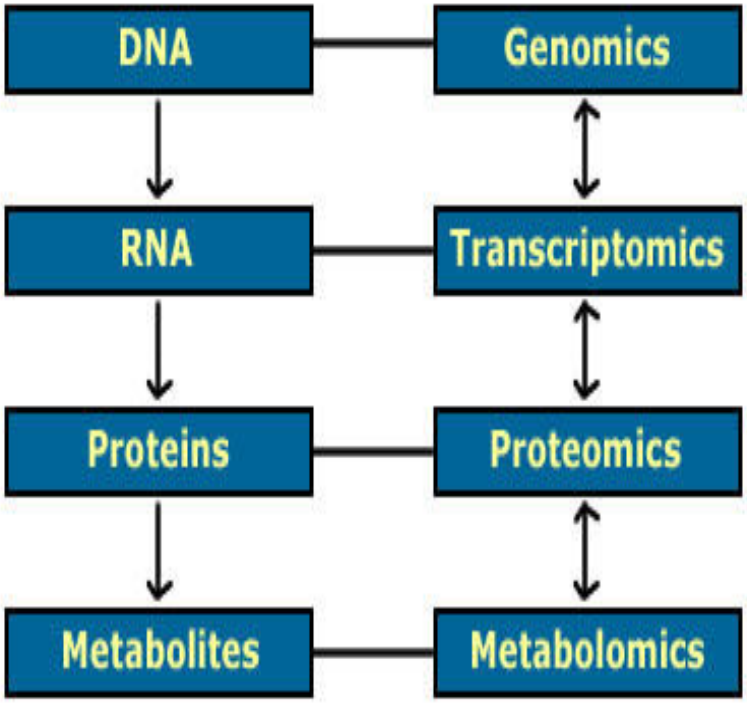


Figure 3.1. General schema showing the relationship from genome to metabolome.

3.2 Materials and methods

3.2.1 Collection of venom glands and total RNA isolation

Female wasps were anaesthetized on ice for several minutes and then shortly rinsed in 70% ethanol. The abdominal organs were gently pulled out with forceps and the reproductive apparatus was placed in 20 µl sterile PBS. Then, the venom gland filaments were dissected, separated from the reservoir and

washed in a drop of sterile ice-cold PBS, put into an Eppendorf tube containing a few microliters of TRIzol® Reagent (Life Technologies) kept on ice and stored at – 80 °C until RNA isolation.

For the experiment, venom gland filaments from 80 female wasps in 300 µl of TRIzol®Reagent were used. After the addition of TRIzol® and before homogenization 5 µl diluted PureLink™ Carrier RNA (25 ng/350 µl) were added to venom glands sample.

The RNA isolation (PureLink™ RNA Micro Kit, Life Technologies) including a treatment on column DNase step (PureLink™DNase) was performed according to the manufacturer's protocol using TRIzol® Reagent with thePureLink™ RNA Micro Kit. Total RNA was eluted from the column with 15 µl RNase-free water.

The concentration of total RNA (199.5 ng/µl) was measured using a Nanodrop and the quality confirmed using a Bioanalyzer and Qubit fluorometer. The yield was 2.4 µg of total RNA from venom glands of 80 female wasps.

3.2.2 RNA-Seq library

RNA-Sequencing (RNA-Seq) is a widely used and powerful method to explore transcriptomes (Sultan*et al.*, 2008; Ozsolak and Milos, 2010).

Before these, the Sanger method was the most utilized thanks to higher efficiency in sequencing, lower amount of radioactivity and usage of toxic chemicals. Due to expensive and not so fast procedure of Sanger sequencing, a new method had to be found. This latter was the next-generation sequencing (Xiong *et al*., 2010; Schuster, 2008) also called high-throughput sequencing or massively parallel sequencing. Next-generation sequencing is parallelized sequencing process, that produces millions of short reads from a single run. These methods are designed to lower the cost of nucleic acids sequencing and to speed up a whole process. RNA-Seq has clear advantages over existing

approaches and is expected to revolutionize the manner in which eukaryotic transcriptomes are analysed (Wang *et al*., 2009). It enables within a single experiment to investigate the expression levels and structure of transcripts without prior knowledge of the transcriptome content (Costa *et al.,* 2010; Richard *et al*., 2010). RNA-Seq uses deep-sequencing technologies allowing us to highlight a more detailed picture with respect to previous techniques (Torres *et al*., 2008; Sultan *et al*., 2008).

In this method, cDNA is fragmented and then sequenced by one of the next-generation sequencers. cDNA derived by reverse transcription of mRNA. A library of mRNAs is chosen by binding poly (A) tails to poly (T) probes on magnetic beads located on a substrate. Each of sequencers and companies have their own protocols and these protocols also differfrom what they are suppose to examine.

To sum it up, the whole process is following:

1. RNA extraction from a sample;
2. RNA library creation;
3. reverse transcription - RNA $\longrightarrow$ cDNA;
4. cDNA fragmentation;
5. sequencing of fragments.

Each molecule, with or without amplification, is then sequenced in a high-throughput manner to obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing).The reads are typically 30-400 bp, depending on the DNA-sequencing technology used. Three main platforms for massively parallel DNA sequencing read production are in reasonably widespread use at present: Illumina/Solexa, 454/Roche and ABI SOLiD.

For this study 2.4 μg of total RNA from venom glands was used to prepare an indexed libraries using IlluminaTruSeq Stranded mRNA and the relative

sequencing is paired-end with reads of length 2x100 bp. Illumina HiSeq1500 platform produced about 40,000,000 reads/RNA sample.

Illumina/Solexa introduced in 2006, is working with reversible terminator chemistry and is based on the concept of "sequencing by synthesis" (SBS) to produce sequence reads of about 32-40 bp from tens of millions of surface amplified DNA fragments simultaneously (Mardis *et al.*, 2008). Illumina® Sequencing technology leverages clonal array formation and proprietary reversible terminator technology for rapid and accurate large-scale sequencing. The innovative and flexible sequencing system enables a broad array of applications in genomics, transcriptomics, and epigenomics.

Sequencing templates are immobilized on a proprietary flow cell surface designed to dispose the DNA in a manner that facilitates access to enzymes while ensuring high stability of surface-bound template and low non-specific binding of fluorescently labeled nucleotides (see www.illumina.com).

Solid phase amplification (Figures 3.2 [1-6]) creates up to 1,000 identical copies of each single template molecule in close proximity (diameter of one micron or less). Because this process does not involve photolithography, mechanical spotting, or positioning of beads into wells, densities on the order of ten million single molecule clusters per square centimeter are achieved.

SBS technology uses four fluorescently-labeled nucleotides to sequence the tens of millions of clusters on the flow cell surface in parallel (Figure 3.2 [7-11]). During each sequencing cycle, a single labeled deoxynucleoside triphosphate (dNTP) is added to the nucleic acid chain. The nucleotide label serves as a terminator for polymerization, so after each dNTP incorporation, the fluorescent dye is imaged to identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide. Since all four reversible terminator-bound dNTPs (A, C, T, G) are present as single, separate molecules, natural competition minimizes incorporation bias. Base calls are made directly from signal intensity measurements during each cycle, which

greatly reduces raw error rates compared to other technologies. The end result is highly accurate base-by-base sequencing that eliminates sequence-context specific errors, enabling robust base calling across the genome, including repetitive sequence regions and within homopolymers.

At the end of the sequencing run (about 4 days), the sequence of each cluster is computed (Figures 3.2 [12]) and subjected to quality filtering to eliminate low-quality reads of between 32 and 40 bp (as specified by the user). A typical run yields about 40-50 millions of such sequences.

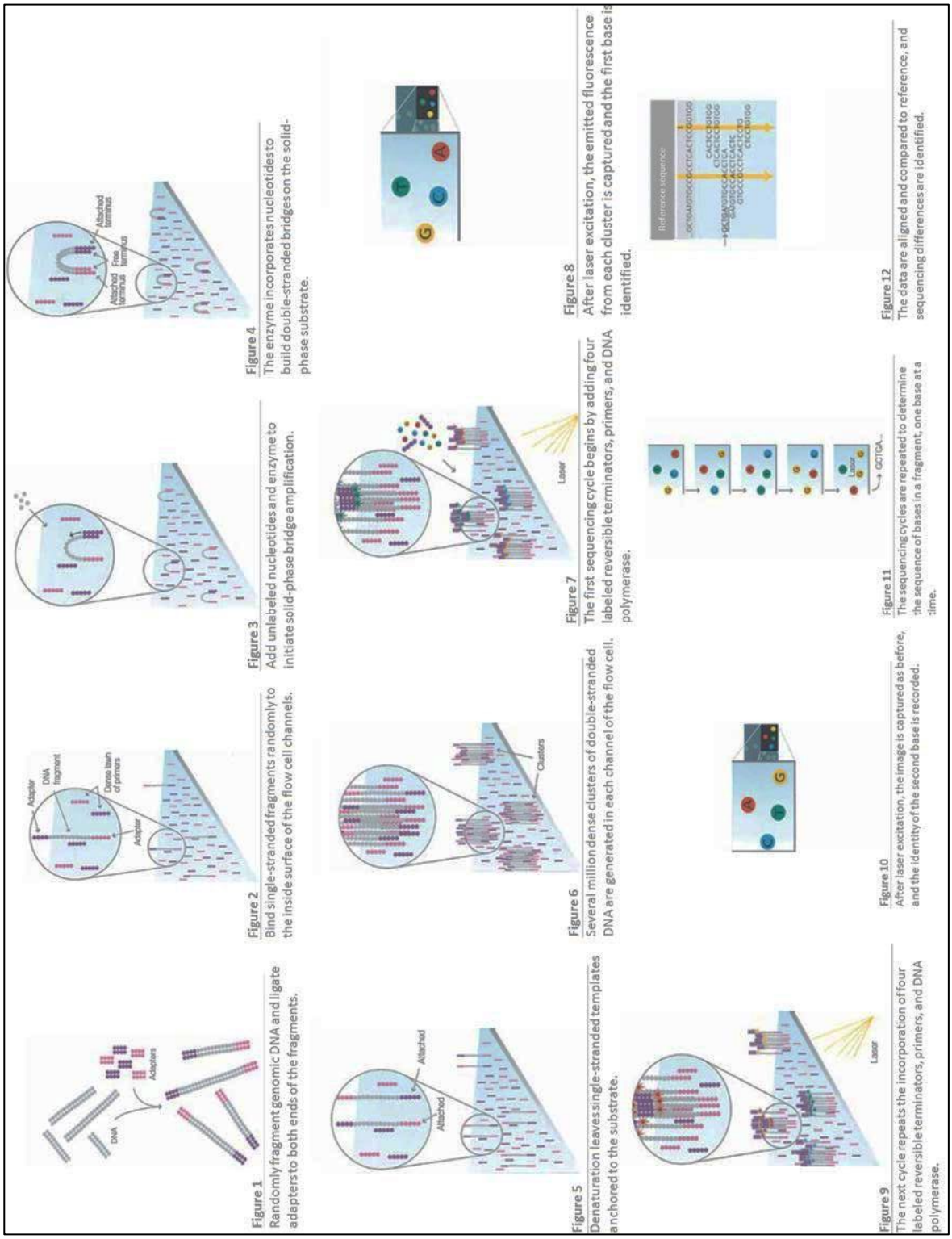


Figure 3.2. Illumina workflow from the website http://www.illumina.com/

3.2.3 Venom extraction for proteomic analysis

Venom from 225 *B. nigricans* females of mixed age were used to proteomic analysis. The reservoirs were separated from venom glands. At least 20 reservoirs were processed each time, and pooled in 10 µl of ice-cold Pringle's solution placed into a glass slide kept on ice. The reservoirs obtained were gently ruptured and the crude venom content immediately collected into an Eppendorf tube. Then, the crude extract was centrifuged at 5000 × g for 5 min, at 4 °C, and the supernatant obtained was stored at -80 °C until used.

The protein content of the venom extracted was quantified by Bradford method; 12 µg of total protein of crude venom extract were used for SDS-PAGE and LC/MS-MS analysis.

3.2.4 SDS-PAGE electrophoresis fractionation and *in situ* digestion

The protein mixture was fractionated by SDS-polyacrilamide gel electrophoresis on Mini Gels (10% acrylamide). After the electrophoresis run, the gel obtained was stained by Coomassie colloidal. Selected protein bands were excised from the gel and washed in 50 mM ammonium bicarbonate pH 8.0 in 50% acetonitrile to a complete destaining.

For each band, the gel pieces were re-suspended in 50 mM ammonium bicarbonate pH 8.0, reduced with 10 mM DTT at 56 °C for 45 min and alkylated with a 55 mM solution of iodoacetamide in the same buffer for 30 min at room temperature in the dark. The excess of reagent was descarded and the gel pieces were washed several times with the buffer, resuspended in 50mM ammonium bicarbonate and incubated with buffer containing 100 ng of trypsin and incubated for 2 hr at 4 °C and overnight at 37 °C.

The supernatant containing the resulting peptides mixture was removed and the gel pieces were re-extracted with acetonitrile. The two fractions were then collected and freeze-dried.

3.2.5 LC/MS-MS analysis

For each sample, the peptide mixture was analyzed by LC/MS-MS using the LC/MSD Trap XCT Ultra (Agilent Technologies, Palo Alto, CA) equipped with a 1100 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture (8 µl in 0.2% formic acid) was first concentrated and washed at 1 µl/min at 4 µl/min in 40 nl enrichment column (Agilent Technologies chip), with 0.1% formic acid as the eluent.

The sample was then fractionated on a C18 reverse-phase capillary column (75 µm x 43 mm in the Agilent Technologies chip) at a flow rate of 300 nl/min, with a linear gradient of eluent B (0.1% formic acid in acetonitrile) in A (0.1% formic acid) from 5 to 60% in 50 min. Elution was monitored on the mass spectrometers without any splitting device. Peptide analysis was performed using data-dependent acquisition of one MS scan (m/z range from 400 to 1600 Da/e) followed by MS/MS scans of the three most abundant ions in each MS scan.

Dynamic exclusion was used to acquire a more complete survey of the peptides by automatic recognition and temporary exclusion (2 min) of ions from which definitive mass spectral data had previously been acquired. Moreover a permanent exclusion list of the most frequent peptide contaminants (keratins and trypsin peptides) was included in the acquisition method in order to focus the analyses on significant data.

3.2.6 Data analysis

Mass spectral data obtained from the LC-MS/MS analyses were used to search in a specific database using an in house version of the Mascot 2.4 (Matrix Science, Boston, MA, USA) software and NCBI database. The specific database, opportunely inserted in house version of the Mascot, is the *B. nigricans* transcripts obtained from bioinformatics analysis previously described. Peptide mass values and sequence information from LC-MS/MS

experiments were used in the MS/MS Ion Search taking into account the Carbamidomethyl-Cys as fixed modification, Met oxidized, Pyro-Carbamidomethyl-Cys (with N-term Carbamidomethyl-Cys) and Pyro-Glu (with N-term E) as variable modifications,1 as max missed cleavages, a precursor ion and a fragment ion mass tolerance of ± 600 ppm and 0.6 Da respectively.

3.2.7 Venom extraction for anionic exchange chromatography

In order to identify bioactive components of the venom responsible for host alterations, *B. nigricans* venom reservoirs were collected from females of mixed age according to Digilio *et al*. (2000), their content was then extracted and fractionated by FPLC.

The whole venom apparatus of adult females, previously frozen for a few min at -20 °C, was pulled out by grasping the ovipositor with forceps, while keeping the abdomen in Pringle's saline solution (Pringle, 1938). The reservoirs were separated from venom glands.

At least 20 reservoirs were processed each time, and pooled in 10 µl of ice-cold Pringle's solution placed into a glass slide kept on ice. The reservoirs obtained were gently ruptured and the crude venom content immediately collected into an Eppendorf tube. Then, the crude extract was centrifuged at 5000 × g for 5 min, at 4 °C, and the supernatant obtained was stored at -80 °C until used.

3.2.8 Polyacrilamide gel electrophoresis of crude venom extract

The presence of proteins in the whole venom extract from 120 female's reservoirs was assessed by SDS-polyacrilamide gel electrophoresis on Nu-PAGE Bis-Tris Mini Gels (1-200 kDa, Invitrogen) and proteins were detected with silver nitrate according to the Blum method (Blum *et al*., 1986).

The quantification of protein content of *B. nigricans* crude venom extract was performed by Bradford assay (1976) using the reagent Dye Reagent Concentrate (Biorad), using bovine serum albumin as standard. 6,5 µl of crude venom (which contains about 770 ng of proteins) were loaded into a 4-12% gradient separating gel and run under 200 V and costant current conditions. The gel was fixed (30% ethanol, 10% acid acetic), stained for 45 min in 0.2 % (w/v) of silver nitrate solution and developed in a solution containing 3 % (w/v) sodium carbonate, 0.025 % (v/v) formaldehyde and 10 mg/ml sodium thiosulfate.

3.2.9 Biological activity of venom proteins

A sample of 120 *B.nigricans* females (120 reservoirs equivalent = RE) was used for extracting venom reservoirs that were processed as described above.

An anionic exchange chromatography of crude venom (120 RE/50 µl of Pringle's solution) was performed using a Mono Q HR 5/5 column by FPLC at room temperature. Running buffer was 50 mM Tris, 50 mM NaCl pH 8.0 and the elution buffer was 50 mM Tris, 1.0 M NaCl pH 8.0.

Chromatographic fractions (41 fractions of 500 µl and 3 fractions of 1 ml) were collected and the protein content determined on 10 µl aliquots by polyacrilamide gel electrophoresis, followed by silver staining.

Fractions were then stored at -80 °C until further use. Biological activity of each chromatographic fraction, was assessed through intra-haemocoelic injection bioassay by injecting 10 µl of each chromatographic fraction in the neck membrane of *S. littoralis* fifth instar larvae (first day), using a 25 µl Hamilton Microsyringe (Hamilton,USA). The bioassay was repeated 3 times for each fraction.

3.3 Results

3.3.1 Reads assembly and bioinformatic analysis

With the purpose of generating a broad vision of the whole transcriptome of venom glands, Illumina paired-end sequencing technology was used to obtained 2 × 100 bp independent reads from either end of a DNA fragment. In this study, a total of 40,000.000 raw sequencing reads with the length of 100 bp were generated.

Trinity (Grabherr *et al*., 2011) was employed for *de novo* assembly of the sequences since no reference genome for *B. nigricans* is available. After quality checking and data cleaning, based on the high quality reads, 63,433 contigs were assembled with an average length of 3,234 bp ranging from 201 to 6,267 bp. On the total of the assembled transcripts, by a Blast X analysis versus the Uniprot Kb reviewed database, 17,228 sequences showed similarity with protein sequences in the database.

3.3.2 Bioactive protein identification

Venoms of parasitic wasps are reputed to have a low content in small proteins and peptides in comparison to venoms of social Hymenoptera (Moreau and Guillot, 2005; Leluk *et al*., 1989). Upon separation of *B. nigricans* venom proteins by SDS-PAGE (Figure 3.3), bands with apparent molecular masses ranging from 15 to 300 kDa had been observed while no bands were seen below 10 kDa.

Figure 3.4 shows the selected protein bands and relative range of molecular weight. In this study, the analyzed bands represent just a part of *B.nigricans* venom proteins; exactly the bands from 1 to 10 and the bands 26 and 27.

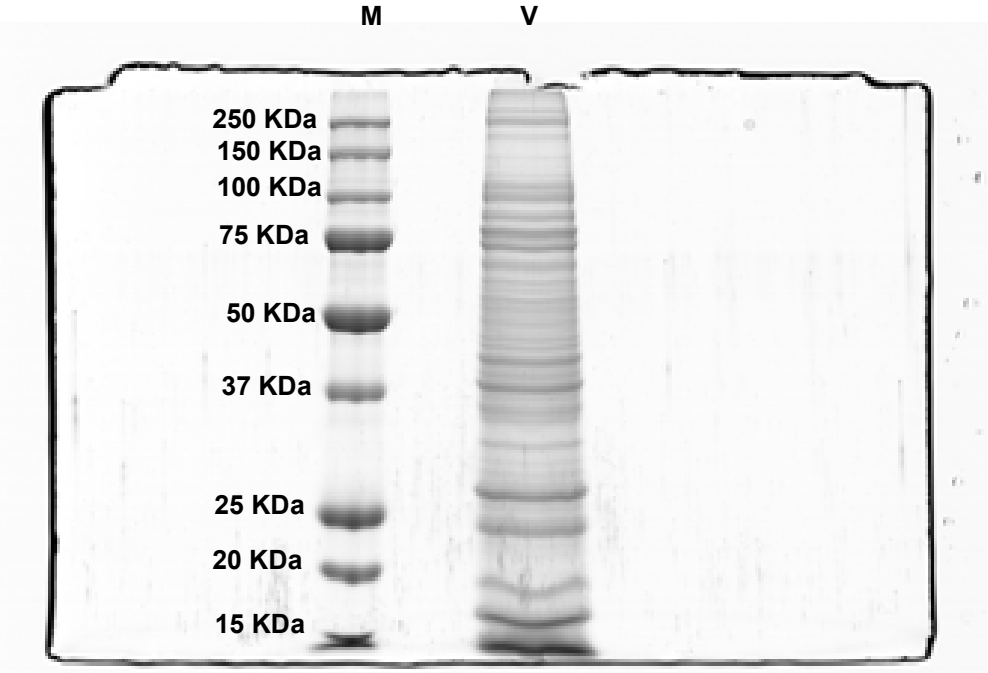


Figure 3.3. SDS-PAGE of *B. nigricans* crude venom extract; M: molecular marker All Blue Biorad, V: 12 µg of crude venom lyophilized and dissolved in 20 µl of Loading Buffer 1X, DTT 100 mM.

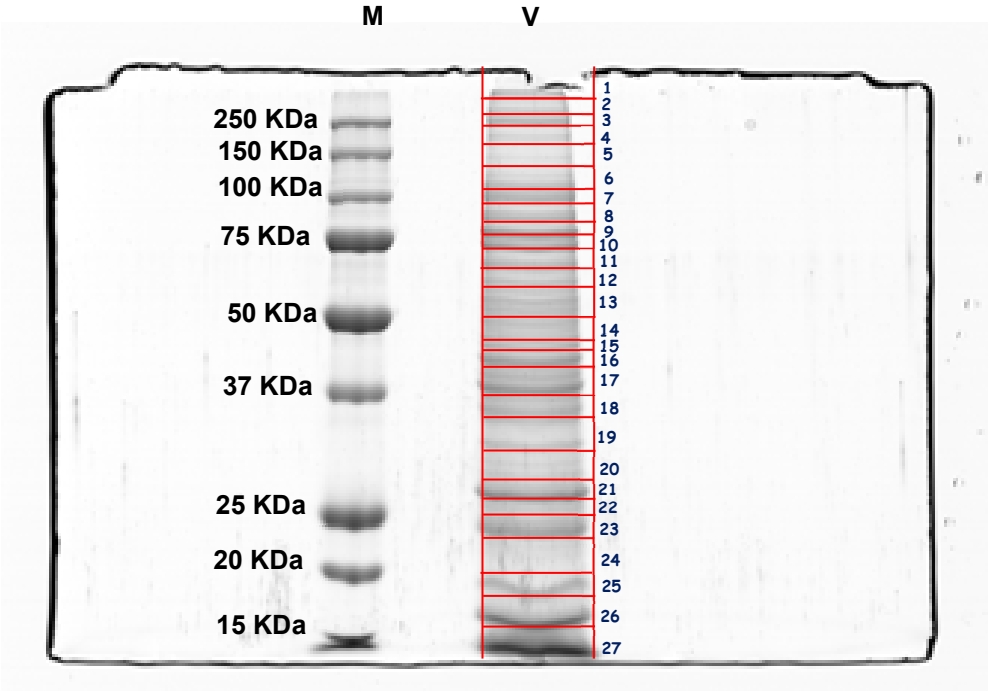


Figure 3.4. Selected protein bands (from 1 to 27) signed in red were excised from the gel and processed for LC/MS-MS analysis.

One band may mask the presence of more than one polypeptide due to the overlapping of proteins with similar molecular masses (see Table 3.1).

The amino acid sequences from LC/MS-MS were matched by Mascot 2.4 software against the database of contings originated from venom gland transcriptome (see the first column of Table 3.1). Matches with the presence of at least two peptides were considered valid. In the second lane the annotations relative to translated sequence of single contig in Swiss Prot database are marked. The matched amino acid sequence present in the contig was sent on Blastp database and the resulting accession number, organism and molecular function annotated (see column 3 and 4 of Table 3.1).

Venom proteins from *B. nigricans* appear to fall into different broad molecular functional categories, including proteases and peptidases, carbohydrate metabolism, esterases, recognition/binding proteins, transport proteins, motor proteins, venom proteins and others. Proteases and peptidases are hydrolases that specifically cleave the peptide bonds found in proteins and peptides. They are essential for the survival of all kinds of organisms, and are encoded for by approximately 2% of all genes (Barrett *et al.*, 2001). They are very common components of venoms, and the ones that we found fall into the categories metallopeptidase, peptidase and hydrolase. Snake venoms contain a variety of metallopeptidases that are highly toxic, resulting in severe bleeding by interfering with blood coagulation and haemostatic plug formation or by degrading the basement membrane or extracellular matrix components of the victims (Matsui *et al.*, 2000). As described by Carvalho *et al*. (2014), metallopeptidase are also representative of *Tityus serrulatus* venom and they are capable of hydrolyzing the neuropeptide dynorphin 1-13 *in vitro*, releasing Leu-enkephalin, which may interact with ion channels and promote indirect neurotoxicity. In *Eulophus pennicornis* a study by Price *et al*. (2010), demonstrated that the injection of recombinant reprolysin-like metallopeptidase (EpMP3) into fifth instar *Lacanobia oleracea* host larvae

resulted in partial insect mortality associated with the moult to sixth instar, with surviving insects showing retarded development and growth. A metallopeptidase present in venom produced by *N. vitripennis* could have an inhibitory function on the coagulation cascade of the host (Danneels *et al*., 2010). Metallopeptidases can function as anti-immune proteins, but they also have roles in the control of development. Amino acid sequence from *B. nigricans* venom proteome have similarity with different metallopeptidase of *N. vitripennis* (Accession Number K7INE4; K7JA72).

An hydrolase sequenced in *B. nigricans* venom shows identity with *N. vitripennis* Calcium-transporting ATPase (Accession Number K7IPT6) a protein involved in the transport of calcium ions. It is worth to note that venom from the ectoparasitic wasp *N. vitripennis* induces cellular injury that appears to involve the release of intracellular calcium stores via the activation of phospholipase C, and culminates in oncotic death, characterized by cell swelling and disruption (Rivers *et al*., 2005).

Another peptide shows identity with a dipeptidyl-peptidase (Accession Number E2B1G2) from *Camponatus floridanus* (carpenter ant) and two aminopeptidase of *Apis cerana* (Accession Number E2BB85; V9IL44).

Another category of proteins characteristic of this venom are enzymes involved in carbohydrate metabolism (Accession Number E2BWP7) similar to Alpha-1,4 glucan phosphorylase of *Harpegnathos saltator* (Jerdon's jumping ant).

Odorant-binding proteins (OBPs) are also present. The OBPs are the main proteins involved in the interaction between odorants and the elements of the sensillar lymph (Pelosi and Maida, 1995; Pelosi, 1996). OBPs include pheromone-binding proteins and general odorant-binding proteins (GOBPs), both of which have molecular masses around 16 kDa and contain six conserved cysteine residues paired in three disulphide bridges (Li *et al.*, 2008). Although Odorant-binding proteins had not yet been described in the

context of insect venoms, GOBPs were also found in *N. vitripennis* venom proteome (de Graaf *et al*., 2010).

It was found a apoliphophorins (Accession Number E2A7K7) and vitellogenin (Accession Number E2C964) which functions as lipid transporters, enables the directed movement of lipids into, out of or within a cell, or between cells.

A protein similar to VG2 (Venom Protein 2) from the parasitoid wasp *Microctonus hyperodae* was found in *B. nigricans* proteome (Accession Number A9YME0). The bioinformatic analysis of the VG2 protein, conducted by Crawford *et al*. (2008), showed it produced peptides matching 11 translated contigs from the *M. hyperodae* EST database. The most significant matches showed high similarity to a *Drosophila melanogaster* protein (CG2206-PB, isoform B), a tyrosine kinase which is absolutely required for its development beyond first instar.

A protein similar to the venom protein Vem 17 from *Chelonus inanitus* (Accession Number E6ZCL5) are present. Vem17 venom protein share sequence similarities with members of a group of proteins similar to protein isoforms A [GenBank:AAF46301.1] and B [GenBank:AAS65275.1] encoded by the lethal G0193 gene from *D. melanogaster*. This group of cystein-rich proteins notably includes several venom and salivary gland proteins of unknown functions reported from various insect species (Vincent *et al*., 2010).

The processing of the selected band 26 (see Figure 3.4) resulted in the identification of a phospholipase A2 (Accession Number C4WRN1).

The stretch of amino acids sequence (116 aa) experimentally found by LC/MS-MS analysis shows 48% of identity with two putative phospholipase A2 of *B. hebetor* present in patented protein sequences database on Blastp (Accession Number CAB42203.1; CAA03259.1). The alignment performed with Blast protein are shown in Figure 3.5.

From the respective sequence of the contig named >comp22364_c1_seq2 (lenght= 715 nucleotides) it was find the ORF sequence by the tool Orf Finder on NCBIsite. A pairwise sequence alignment by ClustalW2 between the ORF sequence (186 aa) and the amino acids sequence (116 aa) shows the 82% of identity (Figure 3.6).

This high percentage of identity allows to state that the stretch of amino acids experimentally found are part of a protein sequence of a phospholipase A2, characteristic of *B. nigricans* venom. In *B. hebetor* the two sequenced phospholipase A2, named BrhTX-1(b) and BrhTX-1(c), are two of the four subunits of the paralysing BrhTX-1 toxin (unpublished sources cited in Weaver *et al*., 2001). These putative enzymes could participate to the diffusion of neurotoxic venomous components in host tissues (Moreau and Guillot, 2005).

Sequence ID: emb|CAB42203.1|Length: 184
Range 1: 65 to 167GenPeptGraphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
103 bits(257)	9e-27	Compositional matrix adjust.	49/103(48%)	68/103(66%)	6/103(5%)

```
Query  19   QTYQGVTNDGYANIYECRCDREFHVCLGQTEHPYLDMAKQLHYDTVHAPCWYKK------  72
            +TY  VTN G++NI+ECRCD  F  CL ++     ++ + LH+D V+ PC++ K
Sbjct  65   ETYGDVTNKGFSNIWECRCDYAFFQCLQRSNGKMKNVVEILHFDVVNTPCYFMKDGRAKI
124

Query  73   SEHTVREKHESLYELVRSKDQHKQWVKDNAHNLLPAELGVPAD  115
            S HTV +KHESLY+L+  KD  K+WV DNA  LLP ELG+ A+
Sbjct  125  SPHTVYDKHESLYQLILHKDNFKEWVHDNAGTLLPRELGIKAE  167
```

Sequence ID: emb|CAA03259.1|Length: 184
Range 1: 65 to 164GenPeptGraphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
101 bits(252)	4e-26	Compositional matrix adjust.	48/100(48%)	66/100(66%)	6/100(6%)

```
Query  19   QTYQGVTNDGYANIYECRCDREFHVCLGQTEHPYLDMAKQLHYDTVHAPCWYKK------  72
            +TY  VTN G++NI+ECRCD  F  CL ++     ++ + LH+D V+ PC++ K
Sbjct  65   ETYGDVTNKGFSNIWECRCDYAFFQCLQRSNGKMKNVVEILHFDVVNTPCYFMKDGRAKI
124

Query  73   SEHTVREKHESLYELVRSKDQHKQWVKDNAHNLLPAELGV  112
            S HTV +KHESLY+L+  KD  K+WV DNA  LLP ELG+
Sbjct  125  SPHTVYDKHESLYQLILHKDNFKEWVHDNAGTLLPQELGI  164
```

Figure 3.5. Alignment sequence protein on Blastp.

```
CLUSTAL 2.1 multiple sequence alignment


comp22364_c1_seq2    MAAITYIIVLVLLIWTSMVSSTFFKPGKWCGPDPNKALQSGSLGERILAICCKIHDECGH 60
amino acid seq       ----------------------------WCGPDPNKALQSGSLGE--------------- 17
                                                 ****************

comp22364_c1_seq2    VIGPKQTYQGVTNDGYANIYECRCDREFHVCLGQTEHPYLDMAKQLHYDTVHAPCWYKKN 120
amino acid seq       ----RQTYQGVTNDGYANIYECRCDREFHVCLGQTEHPYLDMAKQLHYDTVHAPCWYKK- 72
                         :**************************************************

comp22364_c1_seq2    GRAVISEHTVREKHESLYELVRSKDQHKQWVKDNAHNLLPAELGVPADKMWVTLLGMIDI 180
amino acid seq       -----SEHTVREKHESLYELVRSKDQHKQWVKDNAHNLLPAELGVPADK----------- 116
                          ****************************************

comp22364_c1_seq2    NLKTDK 186
amino acid seq       ------
```

Figure 3.6. Pairwise sequence alignment of ORF sequence, obtained from comp22364_c1_seq2 translated sequence and amino acid sequence, experimentally found by LC/MSMS analysis, on ClustalW2.

In general, phospholipases A2 (PLA_2s; EC 3.1.1.4) are a large super-family of lipolytic enzymes that catalyze the hydrolysis of the sn-2 acyl bond of glycerophospholipids to liberate free fatty acids and lysophospholipids (Mingarro *et al*., 1995). PLA2s have important functions in the cellular processes that modulate the release of arachidonic acid and the precursor of eicosanoids of potent inflammatory mediators (Rodriguez de Turco *et al*., 2002) and play a critical role in host defense, atherosclerosis, signal transduction processes, membrane remodeling (Murakami and Kudo, 2002; Dennis, 1994; 1997), and delaying oxidant-induced cell death (Zhao *et al*., 2001). PLA_2s enzymes arise from a variety of sources including the mammalian pancreas, reptile venom, insect venom and synovial fluids (Scott *et al*., 1990).

In *N. vitripennis* PLA 2 is involved in the regulation of fatty acid synthesis and release, which can lead to toxic accumulation, thus inducing death in the long term (Rivers *et al*., 2002). In fact the initial mode of action of the venom may promote the activation of PLC and/or PLA2 as a means to activate fatty

acid synthesis in host fat body (Rivers *et al*., 2002) to facilitate maximum progeny production (Rivers and Denlinger, 1995).

Finally in *B. nigricans* proteome two tipical venom proteins were found: a putative Heat shock 70 kDa protein cognate 5 and cognate 3 (AccessionNumber K7JAU7 and K7ITP5) and an Arginine kinase (Accession Number E1U7W2).

Similarity with structural proteins like putative myosin protein (Accession Number K7J7N4) and other like Hypoxia up-regulated protein 1 (Accession Number E2A725), an Argk (Accession Number G3FP78), an Hsc70Cb (Accession Number S4PX99), a Moesin/ezrin/radixin-like protein 1(Accession Number E2C7G4), an Endoplasmin (Accession Number E2AMV4) and house keeping protein like Aconitate hydratase (Accession Number E2BSG2) were founded.

Table 3.1. Proteins found in the venom of *B.nigricans* with molecular function and relative contig sequence.

ID sequence	SwissProt database Annotations	Database accession number and organism	Molecular functions and /or Protein
Band 1			
comp24426_c0_seq2	Myosin heavy chain P05661 *Drosophila*	K7IRT4 *Nasonia vitripennis*	Motor protein, Myosin
comp23954_c0_seq2	Ca-transp ATPase sarc/ER type P22700 *Drosophila*	K7IPT6 Nasonia vitripennis	Hydrolase
comp18007_c0_seq2	Paramyosin, long form P35415 *Drosophila*	K7J7N4 *Nasonia vitripennis*	Motor activity
comp22102_c0_seq1	No hits found		
Band 2			

comp24426_c0_seq4	Myosin heavy chain P05661 *Drosophila*	K7IRT4 *Nasonia vitripennis*	Motor protein, Myosin
comp24964_c0_seq1	Apolipophorins Q9U943 *Locusta migratoria*	E2A7K7 *Camponotus floridanus*	lipid transporter activity
comp33033_c0_seq1	Apolipophorins Q9U943 *Locusta migratoria*	E2A7K7 *Camponotus floridanus*	lipid transporter activity
Band 3			
comp24426_c0_seq4	Myosin heavy chain P05661 *Drosophila*	K7IRT4 *Nasonia vitripennis*	Motor protein, Myosin
comp24308_c0_seq1	Vitellogenin-6 Q94637 *Oscheius tipulae*	E2C964 *Harpegnathos saltator*	lipid transporter activity
comp23954_c0_seq1	Ca-transp ATPase sarc/ER type Q7PPA5 *Anopheles gambiae*	K7IPT6 *Nasonia vitripennis*	Hydrolase
comp24673_c0_seq1	Clathrin heavy chain P29742 *Drosophila*	V9IKI6 *Apis cerana*	Structural molecule activity
comp18379_c0_seq1	Arginine kinase O61367 *Apis mellifera*	G3FP78 *Bombus hypocrita*	ATP binding
comp22460_c0_seq1	Pyruvate kinase O62619 *Drosophila*	E2AJ75 *Camponotus floridanus*	pyruvate kinase activity
Band 4			
comp24426_c0_seq2	Myosin heavy chain P05661 *Drosophila*	K7IRT4 *Nasonia vitripennis*	Motor protein, Myosin
comp23954_c0_seq2	Ca-transp ATPase sarc/ER type P22700 *Drosophila*	K7IPT6 *Nasonia vitripennis*	Hydrolase
comp18379_c0_seq2	Arginine kinase O61367 *Apis mellifera*	G3FP78 *Bombus hypocrita*	kinase activity
comp26660_c0_seq1	Hypoxia up-regulated protein 1 Q566I3 *Xenopus laevis*	E2A725 *Camponotus floridanus*	ATP binding
Band 5			
comp24426_c0_seq2	Myosin heavy chain P05661 *Drosophila*	K7IRT4 *Nasonia vitripennis*	Motor protein, Myosin

comp21536_c0_seq1	Pyruvate carboxylase Q05920 *Mus musculus*	E2AWL4 *Camponotus floridanus*	ATP/DNA binding, pyruvate carboxylase activity
comp23954_c0_seq1	Ca-transp ATPase sarc/ER type Q7PPA5 *Anopheles gambiae*	K7IPT6 *Nasonia vitripennis*	Hydrolase
comp25466_c0_seq1	Ubq-like modifier-activ Enz 1 P22314 *Homo sapiens*	E2C890 *Harpegnathos saltator*	small protein activating enzyme activity, ATP binding
comp18379_c0_seq1	Arginine kinase O61367 *Apis mellifera*	E1U7W2 *Pteromalus puparum*	ATP binding
comp27249_c0_seq1	Aminopeptidase N P91887 *Plutella xylostella*	E2BB81 *Harpegnathos saltator*	aminopeptidase activity
comp22165_c0_seq2	Lysosomal alpha-mannosidase Q8VHC8 *Cavia porcellus*	K7J2I6 *Nasonia vitripennis*	alpha-mannosidase activity, carbohydrate binding
comp12616_c0_seq1	Thyrotropin-releasing Horm-degr Enzyme Q9UKU6 *Homo sapiens*	K7INE4 *Nasonia vitripennis*	metallopeptidase activity
Band 6			
comp18007_c0_seq2	Paramyosin, long form P35415 *Drosophila*	K7J7N4 *Nasonia vitripennis*	Motor activity
comp23954_c0_seq1	Ca-transp ATPase sarc/ER type Q7PPA5 *Anopheles gambiae*	K7IPT6 *Nasonia vitripennis*	Hydrolase
comp23143_c0_seq2	2-oxoglutarate dehydrogenase Q148N0 *Bos taurus*	F4X6J5 *Acromyrmex echinatior*	oxoglutarate dehydrogenase (succinyl-transferring) activity, thiamine pyrophosphate binding
comp13820_c0_seq1	Leucyl-cystinyl aminopeptidase P97629 *Rattus norvegicus*	E2BB85 *Harpegnathos saltator*	aminopeptidase, metallopeptidase activity, zinc ion binding
comp23948_c0_seq1	Vinculin O46037 *Drosophila*	E2BTN6 *Harpegnathos saltator*	structural molecule activity
comp20972_c0_seq1	Presequence protease Q5JRX3 *Homo Sapiens*	E9IPF6 *Solenopsis invicta*	catalytic activity

comp18242_c0_seq1	97 kDa heat shock protein Q06068 *Strongylocentrotus purpuratus*	S4PX99 *Pararge aegeria*	ATP binding
comp24713_c0_seq1	Staphylococcal nuclease domain-cont P 1 Q863B3 Bos taurus	V9IGS7 *Apis cerana*	hydrolase activity, acting on ester bonds
Band 7			
comp17149_c0_seq1	Glutamyl aminopeptidase Q07075 *Homo Sapiens*	E2BB85 *Harpegnathos saltator*	aminopeptidase, metallopeptidase activity, zin ion binding
comp18007_c0_seq2	Paramyosin, long form P35415 *Drosophila*	K7J7N4 *Nasonia vitripennis*	Motor activity
comp24009_c1_seq3	Alpha-actinin, sarcomeric P18091 *Drosophila*	E2ACU8 *Camponotus floridanus*	calcium ion binding
comp23954_c0_seq1	Ca-transp ATPase sarc/ER type Q7PPA5 *Anopheles gambiae*	K7IPT6 *Nasonia vitripennis*	Hydrolase
comp24430_c0_seq1	Endoplasmin Q95M18 *Bos taurus*	E2AMV4 *Camponotus floridanus*	ATP binding
comp22940_c0_seq2	Filamin-A Q9VEN1 *Drosophila*	E2BBG1 *Harpegnathos saltator*	Filamin-C gene: EAI_04416
comp23143_c0_seq1	2-oxoglutarate dehydrogenase Q60HE2 *Macaca fascicularis*	E2AHW3 *Camponotus floridanus*	oxoglutarate dehydrogenase (succinyl-transferring) activity, thiamine pyrophosphate binding
comp13820_c0_seq1	Leucyl-cystinyl aminopeptidase P97629 *Rattus norvegicus*	E2BB85 *Harpegnathos saltator*	aminopeptidase, metallopeptidase activity, zin ion binding
comp26029_c0_seq1	Glycogen phosphorylase Q9XTL9 *Drosophila*	E2BWP7 *Harpegnathos saltator*	carbohydrate metabolic process
comp24426_c0_seq3	Myosin heavy chain P05661 *Drosophila*	K7IRT4 *Nasonia vitripennis*	Motor protein, Myosin
comp22531_c0_seq2	Glycogen phosphorylase Q9XTL9 *Drosophila*	E2BWP7 *Harpegnathos saltator*	carbohydrate metabolic process

comp21027_c0_seq1	Puromycin-sensitive aminopeptidase Q11011 *Mus musculus*	V9IL44 *Apis cerana*	aminopeptidase activity
Band 8			
comp23136_c0_seq1	Aconitate hydratase Q99KI0 *Mus musculus*	E2BSG2 *Harpegnathos saltator*	aminopeptidase activity
comp18078_c0_seq1	No hits found	A9YME0 *Microctonus hyperodae*	Venom protein 2
comp14282_c0_seq1	No hits found	A9YME0 *Microctonus hyperodae*	Venom protein 2
Band 9			
comp24894_c0_seq1	No hits found	E1ZZB9 *Camponotus floridanus*	putative uncharacterized protein
comp24599_c1_seq1	Endothelin-conver Enzyme-like 1 O95672 *Homo sapiens*	W8BVC8 *Ceratitis capitata*	metalloendopeptidase activity
comp23895_c0_seq1	Endothelin-converting Enzyme 1 P42892 *Homo sapiens*	K7JA72 *Nasonia vitripennis*	metalloendopeptidase activity
comp24555_c1_seq1	Moesin/ezrin/radixin homolog 1 B0WYY2 *Culex quinquefasciatus*	E2C7G4 *Harpegnathos saltator*	Moesin/ezrin/radixin-like protein 1
comp18045_c0_seq1	Heat shock 70 kDa P cognate 3 P29844 *Drosophila*	E2AY69 *Camponotus floridanus*	ATP binding
comp24916_c0_seq1	No hits found	A9YME0 *Microctonus hyperodae*	Venom protein 2
comp23518_c0_seq1	Dipeptidyl peptidase 3	E2B1G2	dipeptidyl-peptidase activity
	Q9VHR8 *Drosophila*	*Camponotus floridanus*	
comp23136_c0_seq1	Aconitate hydratase Q99KI0 *Mus musculus*	E2BSG2 *Harpegnathos saltator*	Aconitate hydratase
comp20936_c0_seq1	No hits found	E9IV56 *Solenopsis invicta*	putative uncharacterized protein
comp14043_c0_seq1	No hits found	E6ZCL5 *Chelonus inanitus*	Venom protein Vem 17

comp17149_c0_seq1	Glutamyl aminopeptidase Q07075 *Homo sapiens*	E2BB80 *Harpegnathos saltator*	aminopeptidase activity
comp23189_c0_seq1	No hits found	E1ZZB9 *Camponotus floridanus*	uncharacterized protein
comp24195_c0_seq2	Calpain-B Q9VT65 *Drosophila*	V9I6P7 *Apis cerana*	calcium-dependent cysteine-type endopeptidase activity, calcium ion binding
Band 10			
comp24842_c0_seq1	No hits found	E2B3J2 *Harpegnathos saltator*	putative uncharacterized protein
comp20898_c0_seq1	No hits found	E2B3J2 *Harpegnathos saltator*	putative uncharacterized protein
comp24912_c0_seq1	Heat shock 70 kDa P cognate 4 Q9U639 *Manduca sexta*	K7IQ77 *Nasonia vitripennis*	ATP binding
comp23743_c2_seq1	V-type proton ATPase catal. sub. A Q2TJ56 *Aedes albopictus*	K7INR1 *Nasonia vitripennis*	ATP binding, proton-transporting ATPase activity, rotational mechanism
comp18045_c0_seq1	Heat shock 70 kDa protein cognate 3 P29844 *Drosophila*	*K7ITP5* *Nasonia vitripennis*	ATP binding
comp24916_c0_seq1	No hits found	A9YME0 *Microctonus hyperodae*	Venom protein 2
comp22969_c0_seq1	Heat shock 70 kDa protein cogn 5 P29845 *Drosophila*	K7JAU7 *Nasonia vitripennis*	ATP binding
comp24894_c0_seq1	No hits found	E1ZZB9 *Camponotus floridanus*	putative uncharacterized protein
comp22390_c0_seq1	No hits found	V9I8K5 *Apis cerana*	uncharacterized protein
Band 26			
comp25028_c0_seq1	Cofilin/actin-depolymer factor homolog P45594 *Drosophila*	K7IPR6 *Nasonia vitripennis*	actin filament depolymerization

comp22364_c1_seq2	Phospholipase A2 (PA3A/PA3B/PA5) P16354 *Heloderma suspectum*	C4WRN1 *Acyrthosiphon pisum*	calcium ion binding, phospholipase A2 activity
comp16748_c0_seq1	Superoxide dismutase [Cu-Zn] P28755 *Ceratitis capitata*	K7IYG5 *Nasonia vitripennis*	superoxide dismutase activity
comp24955_c0_seq1	Peptidyl-prolyl cis-trans isomerase P54985 *Blattella germanica*	K7J0P3 *Nasonia vitripennis*	peptidyl-prolyl cis-trans isomerase activity
Band 27			
comp24796_c0_seq1	No hits found	T1H8Z3 *Rhodnius prolixus*	odorant binding
comp18097_c0_seq1	No hits found	B4HT21 *Drosophila*	transporter activity
comp17530_c0_seq1	No hits found	B5DI21 *Drosophila pseudoobscura*	gene: Dpse\GA25896
comp24825_c0_seq1	No hits found	W8C879 *Ceratitis capitata*	odorant binding
comp16285_c0_seq1	Cytochrome c-2 P84029 *Drosophila*	K7IXL7 *Nasonia vitripennis*	electron carrier activity

3.3.3 Identification of a bioactive chromatographic fraction

The electrophoresis of crude venom shows a complex pattern of proteins (Figure 3.7). The molecular mass of venom proteins were estimated by comparison with the molecular mass of different standard proteins.

Four chromatographic fractions on the total, shows proteic bands on gel and a temporary paralysis was observed when a fraction containing a protein of about 17 kDa was injected.

The paralysis was observed in all three larvae injected with this fraction, and lasted about 2 hours (Figure 3.8a, b).

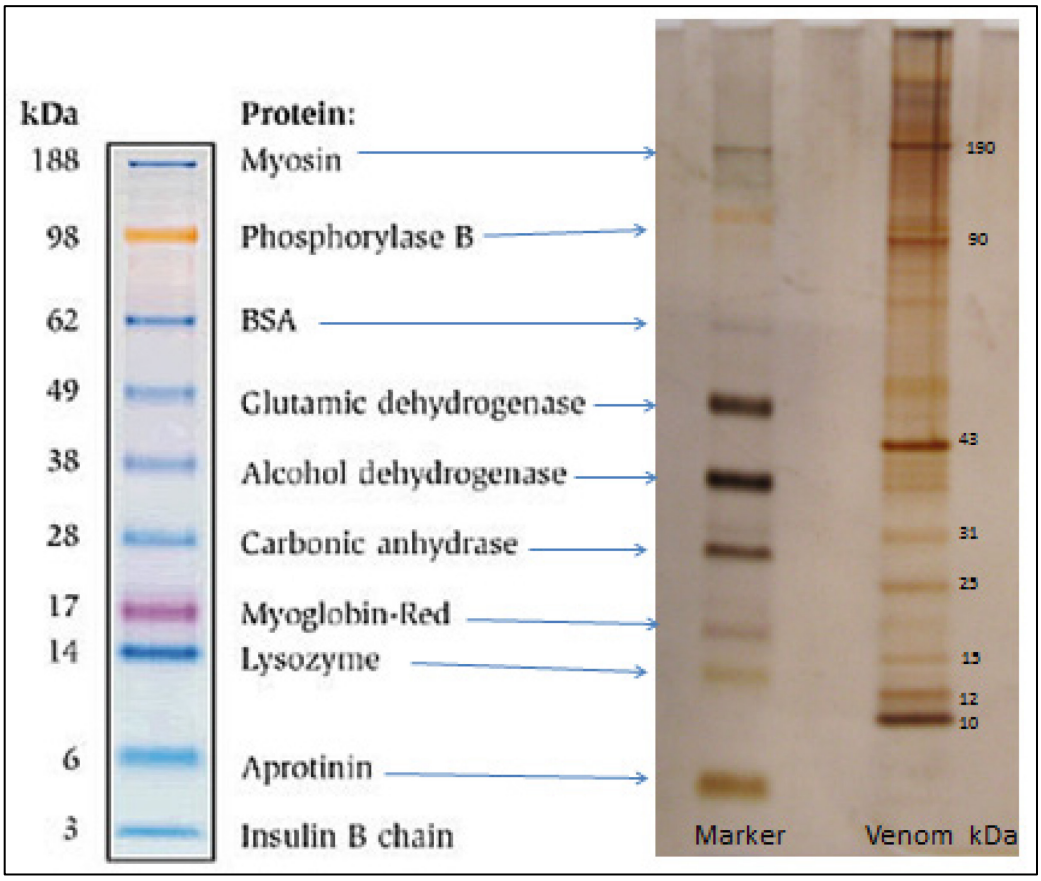


Figure 3.7. SDS-Polyacrilamide gel electrophoresis of crude venom extracts of *B. nigricans.*

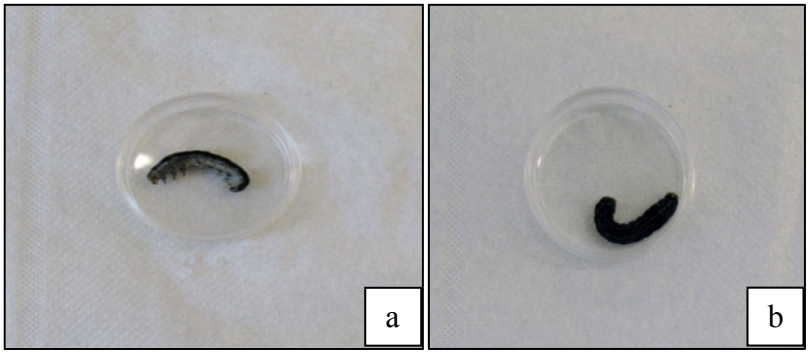


Figure 3.8. (a) Paralyzed larva after injection of the 17 kDa venom protein; (b) recovered larva 2 hours after protein injection.

3.4 Discussion and conclusion

The results of this part of the thesis, obtained through the combined approaches of transcriptomics and proteomics, provide an overview of the main components of the venom and the functional role played by a bioactive

component isolated from *B. nigricans* venom crucial for the regulation of the host *S. littoralis*.

This study revealed a complex and rich composition of *B. nigricans* venom. The presence of specific proteins which are involved in several metabolic pathways is well in tune with the role of venom in enhancing and supporting the progenies' development. Proteins with lipid transport activity may be involved in the mobilization of storage nutrients for the development of parasitoids larvae. Proteins involved in carbohydrate metabolism are present in this venom and their function could be the release of carbohydrates in hemolymphatic cavity to enhance the host suitability.

Calcium-binding proteins are also present and are typical of several venomous organisms; they are involved in the stimulus-secretion coupling mechanisms in secretory glands (Gonçalves *et al*., 1997). Deregulation of intracellular calcium levels can result in irreversible injury and has been implicated in several disease conditions (Yáñez *et al*., 2012). Thus, calcium homeostasis, the control of intracellular Ca^{2+} concentration, is very tightly regulated. As described in other parasitoid, *B. nigricans* venom may thus stimulate calcium release to determine host cell damage (e.g. fat body cells) and increased availability of nutrients.

In venomic research it is not uncommon to find so-called 'venom trace elements' with only a local function in the venom duct or reservoir or released by leakage of the gland tissue (de Graaf *et al.*, 2010). The finding of actin and myosin could indicate that some cytoplasmatic proteins might have been released while the reservoir were spun-down during venom collection.

Finally, characteristic allergenic proteins like heat shock 70 kDa protein and arginine kinase were found. This latter enzyme, in a spider wasp *Cyphononyx dorsalis*, exerts paralytic activity in the host with the same characteristic symptoms as the crude venom (Yamamoto *et al*., 2007). However, arginine

kinase usually acts in energy metabolism in invertebrates (Wu *et al*., 2007) and as an allergen (Binder *et al*., 2001; Garcìa-Orozco *et al*., 2007).

The most interesting protein found was the enzyme phospholipase A2, that probably represents the main toxin of this venom. This protein was found in a band of about 17 kDa, as similarly reported for the closely related species *B. hebetor,* which do also has in the venom a phospholipase A2, having paralytic activity and the same molecular mass.

Additionally, it's worth to note that the chromatographic fraction biologically active, that causes a temporary paralysis in *S. littoralis* larva, contains a protein band of about 17 kDa, currently being characterized. While the precise functions of *B. nigricans* venom phospholipase A2 remain to be established, the successful cloning of its full-length cDNA will allow the production of the recombinant protein and the unraveling of its role in host-regulation process.

CHAPTER 4: Production of silver nanoparticles and their effects against *Hylastinus obscurus* (Marsham) (Coleoptera: Curculionidae)

4.1 Introduction

Nanotechnology, coined by Taniguchi (1974), refers to all technological developments performed at nanoscale (10^{-9} m).

Nanotechnology is a promising field of interdisciplinary research. It opens up a wide array of applications in various fields like pharmaceutics, electronics and agriculture. The potential uses and benefits of nanotechnology in the agricultural field are enormous. These include management of insect pests through the formulation of nanomaterials-based insecticides. Indeed, traditional strategies like integrated pest management are in some cases insufficient, and application of chemical pesticides has well known adverse effects on animals and human beings apart from the decline in soil fertility. Therefore, nanotechnology would provide green and efficient alternatives for the management of insect pests in agriculture without harming the nature (Ragaei and Sabry, 2014).

Nanoparticles may thus represent a new generation of insecticides to exploit in the pest management of the future (Battacharyya *et al.*, 2010).

Antimicrobial properties of nanopartciles of metal ions are already famous (Law *et al*., 2008; Kim *et al*., 2007; Silver, 2003; Mohan Kumar *et al*., 2012).

Nanoparticles can be produced by a sort of green synthesis from plants, plant products, bacteria, fungi, yeasts, algae and viruses (Thakkar *et al*., 2010). In comparison to microorganisms, phytosynthesis method is devoid of complex and multistep processes like microbial isolation, culturing, maintenance etc., and is also very rapid and cost effective that can be easily scaled up for bulk production of nanoparticles (Shankar *et al*., 2004). Moreover, it has been

shown that the rate of nanoparticle synthesis is faster using plants than microbes and the produced nanoparticles are more stable (Iravani, 2011).

It can be said that phytosynthesis is truly a "green" synthesis way in comparison to other known methods of nanoparticle synthesis.

The main underlying principle behind the biogenesis of nanoparticles is the reduction of metal ions by various biomolecules like enzymes/proteins, amino acids, polysaccharides and vitamins present in the organism (Pulikotil *et al*., 2013). In particular, the production of metal nanoparticle by plants is due to the presence of phytochemicals like flavones, ketones, aldehydes, amides and carboxylic acids which reduce metal cations into metal nanoparticles (Pulikotil *et al*.,2013), acting as powerful reducing agents. In fact, the plant extract has the potential to reduce Ag^{+} ions into silver nanoparticles from a solution of silver nitrate (Sadowski Z., 2013). The exact mechanism underlying the formation of nanoparticles has not been identified yet. However on the basis of kinetic studies with FT-IR (Fourier transform infrared) spectrometry and cyclic voltammetry, Begum *et al*. (2009) suggested that polyphenols and flavonoids present in the leaves of the tea plant could be responsible for the synthesis of nanoparticles of Ag and Au. In fact, a leaf extract free of polyphenols and flavonoids did not form nanoparticles. Interestingly, Narayanan and Sakthivel (2011) reported that plant mediated synthesis cannot occurr by enzymes, as generally the plant extract is heated up to 90 ºC during the synthesis process. According to them, phytochemicals such as phenolics, terpenoids, sesquiterpenes and flavonoids and their functional groups present in these phytochemicals are involved in the reduction and capping of nanoparticles (Figure 4.1).

The most largely used metal nanoparticles are silver and gold nanoparticles. In insects, the administration of silver nanoparticles (AgNPs) induces physiological, behavioural and biological alterations such as cuticolar

demelanization (Armstrong *et al*., 2013) and even mortality (Kalantari *et al*., 2012).

However, the exact mechanism of AgNPs action and toxicity has not been determined.

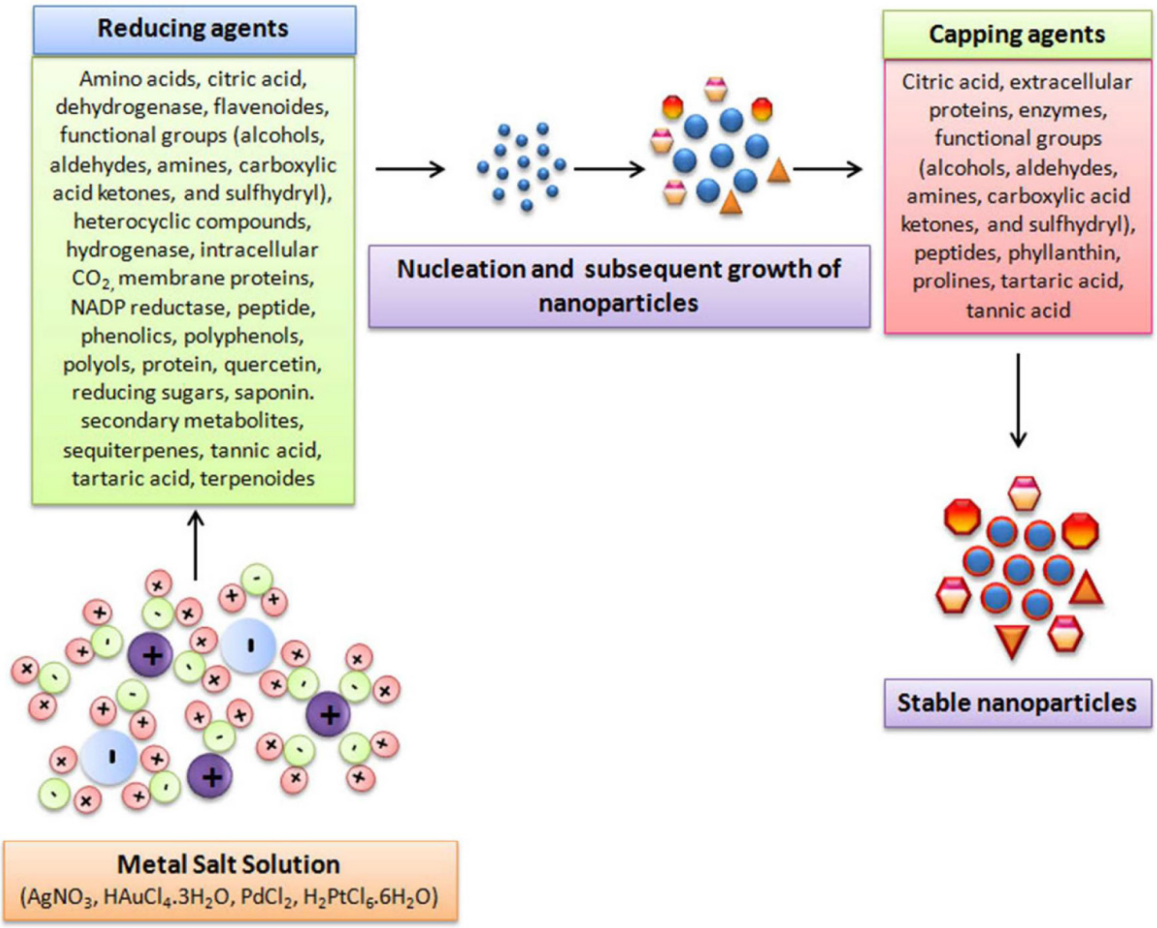


Figure 4.1. Schematic diagram showing the mechanism of biogenic metallic nanoparticles synthesis (from Akhtar *et al*., 2013).

4.2 Aim of the work

The aim of this part of thesis was to verify the effects of silver nanoparticles against the red clover root borer, *Hylastinus obscurus* (Marsham) (Coleoptera: Curculionidae). This work was carried out in Chile, in the Laboratorio de

Química Ecológica (Departamento de Ciencias Químicas y Recursos Naturales, Universidad de La Frontera, Temuco).

This curculionid causes a significant reduction in the production levels and the persistence of red clover (*Trifolium pratense* L.) fields because both larvae and adults bore and feed within the roots (Aguilera *et al*., 1996). Pesticides do not successfully control *H. obscurus* infestations (Aguilera *et al*., 1996); therefore, alternative strategies for controlling this insect are necessary. At now, the use of semiochemicals may provide a new alternative to reduce the damage caused by this pest (Parra *et al*., 2013).

4.3 Materials and methods

4.3.1 Nanoparticles formation

Two different plant samples were collected for the synthesis of silver nanoparticles: leafs from *Hypericum perforatum* and *Galega officinalis*, two wild plants that grow abundantly in suburban areas of Southern Chile.

The whole plants were collected in the summer 2014 and frozen at -80 °C until use. The aqueous extract of each frozen plant was prepared by boiling 10 g of the material in 100 ml of deionized water. After 5 min, the macerate was filtered on Whatmann filter paper #1.

800 ml of 1 mM $AgNO_3$ solution was added to 8 ml of plant extract and for a bioreduction process silver nanoparticles were formed. The solution was adjusted to pH 10 by the addition of a few drops of 6 M NaOH.

The solution was then incubated for 4 hours at room temperature.

At the end of the incubation time, to confirm the production of silver nanoparticles, the absorption spectra were measured in the range of 200-800 nm wavelengths, using a Genesis Ios UV-vis spectrophotometer. Deionized water was used as blank. The absorbance profile at 415 nm confirmed the presence of silver nanoparticles.

The mean particle diameter of silver nanoparticles was assessed with a Malvern Zetasizer Nanosystem (Worcestershire, UK).

The aqueous suspension of the synthesized silver nanoparticles was filtered through a 0.22 μm syringe driven filter unit and the size of silver nanoparticles was measured by a Malvern Zetasizer Nano series compact scattering spectrometer, exploiting the principle of dynamic light scattering (DLS) technique. The extract from each plant was lyophilized, weighed and resuspended in deionized water to a final concentration of 9.2 g/L and 36.4 g/L for *H. perforatum* and *G. officinalis* extracts respectively.

4.3.2 Insecticide bioassay

Adults of *H. obscurus* were collected from red clover plants in INIA-Carillanca. The insects were removed from the roots and maintained in Petri dishes on little pieces of red clover roots at 18-22 °C and 18 L:6 D light regimen. To perform the feeding bioassays, insects were removed from the root pieces 24 h before each bioassay according with Ortega *et al*. (2014).

The feeding performance of *H. obscurus* adults was evaluated in a no-choice test using an artificial diet according to the methodology described by Faccoli and Schlyter (2007). Approximately 400 μL (density = 1.05 g/ml) of a diet composed by 2% (w/v) cellulose, 2.6% (w/v) glucose, 4.3% (w/v) starch and 3.5% (w/v) agar (in water) was added into a transparent glass tube (6 mm diameter × 25 mm length). Then, 25 μL of each AgNPs solution were added separately at two different concentrations (9200 and 36400 ppm) into the liquid diet. The suspension was then carefully mixed and left solidifies at room temperature.

Subsequently one previously weighed (initial weight: iw) *H. obscurus* adult was introduced into each tube, which was then closed with plastic parafilm.

For each treatment consition, 15 to 20 insects were used. Two controls were prepared: insects fed with artificial diet alone and artificial diet supplemented

with 25 μL of 1 mM $AgNO_3$. Each insect was allowed to feed for 11 days in darkness at room temperature, evaluating the daily mortality and the presence of eventual tunnels in the diet. Survivors were weighed (final weight: fw) at the end of the experiment. The feeding performance was evaluated in terms of weight increase (%), where: weight increase (%) = (fw-iw) × 100/fw. The percentage of insect mortality for each treatment (% mortality = number of dead insects/total insects × 100) and the percentage of insects that have excavated tunnels for each day (% tunnels/day = number of vials with tunnels/number of alive insects × 100) were also evaluated.

4.4 Statistical analysis

Results of feeding bioassay were analyzed by Kruskal-Wallis tests ($P \leq 0.05$), and statistical differences among groups were determined by Dunn's Multiple Comparison Test. The % of tunnels was analyzed by nonparametric Friedman test followed by Dunn's Multiple Comparison Test ($P \leq 0.05$) using GraphPad Prism 5.

4.5 Results

4.5.1 Nanoparticles formation

As showed by Durán *et al*. (2007) the formation of nanoparticles was confirmed by the appearance of a brown color after 4 h of incubation at room temperature. Moreover, a factor affecting the formation of metal nanoparticles is the pH value of plant extract. It has been hypothesized that at higher pH, the availability of a large number of negatively charged groups facilitates the formation of a greater number of Ag^+ to bind and subsequently to form a large number of nanoparticles with a low diameter (Akhatar *et al*., 2013).

The average diameters of silver nanoparticles, assessed with a Malvern Zetasizer Nanosystem, from *H. perforatum* and *G. officinalis* were 85 and 100 nm, respectively.

4.5.2 Bioassays with nanoparticles produced with *H. perforatum* extracts

Feeding bioassay showed no statistically differences in weight increase was observed among the different treatments t (controls, 9200 and 36400 ppm) (Figure 4.2).

The percentage of tunnels excavated in the diet was lower when individuals fed on diet supplemented with nanoparticles at 9200 ppm than the controls (Figure 4.3a).

Individuals fed on diet with nanoparticles at 36400 ppm showed no difference in the excavation behavior in respect to control insects fed with diet supplemented with silver nitrate and a slight difference in respect to control insects fed with diet alone (Figure 4.3b).

The mortality in insects fed on diet with nanoparticles at 9200 ppm was 10% higher than controls (Figure 4.4a), while no significant difference in insects treated with nanoparticles at 36400 ppm was detected (Figure 4.4b).

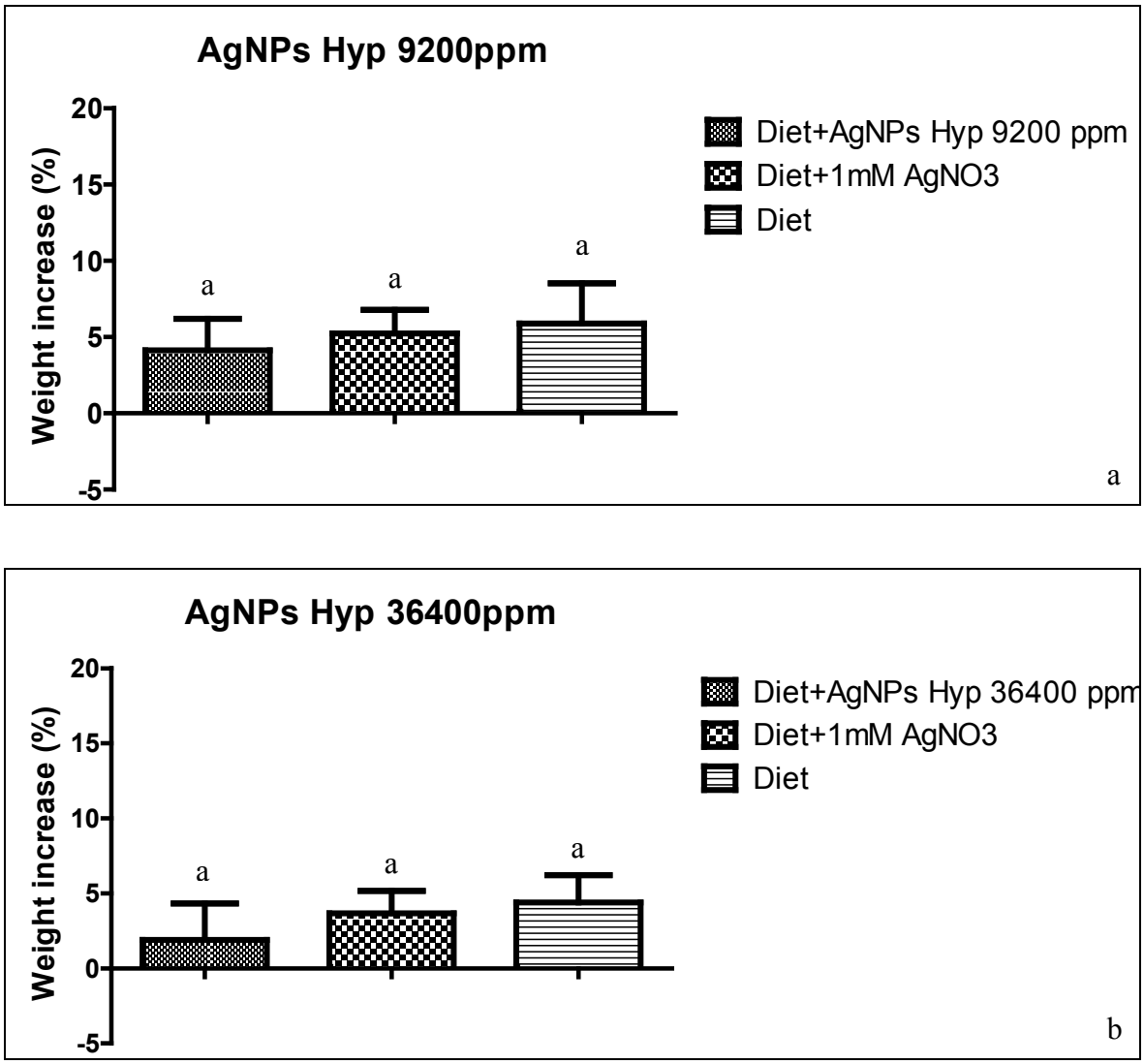
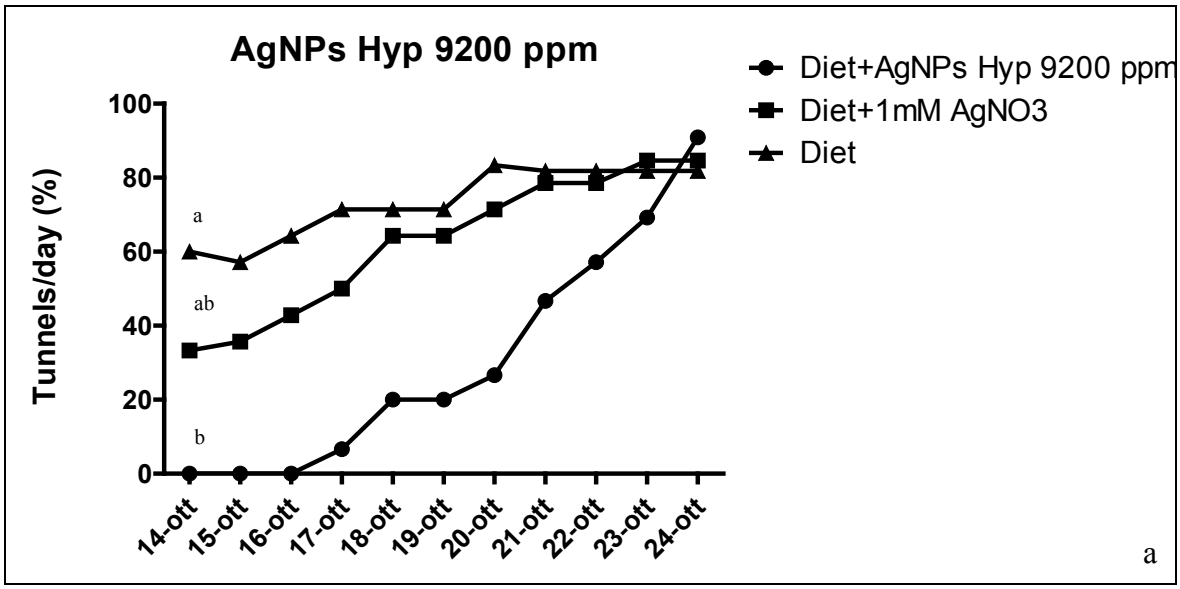


Figure 4.2. Weight increases (% ± SEM) of *H. obscurus* individuals fed on artificial diet supplemented with silver nanoparticles from *H. perforatum* extract (AgNPs Hyp) at 9200 and 36400 ppm. Controls were fed with artificial diet alone or artificial diet supplemented with 1 mM $AgNO_3$. Different letters indicate significant differences between treatments based on a nonparametric Kruskal-Wallis test followed by a Dunn's Multiple Comparison Test ($P \leq 0.05$).



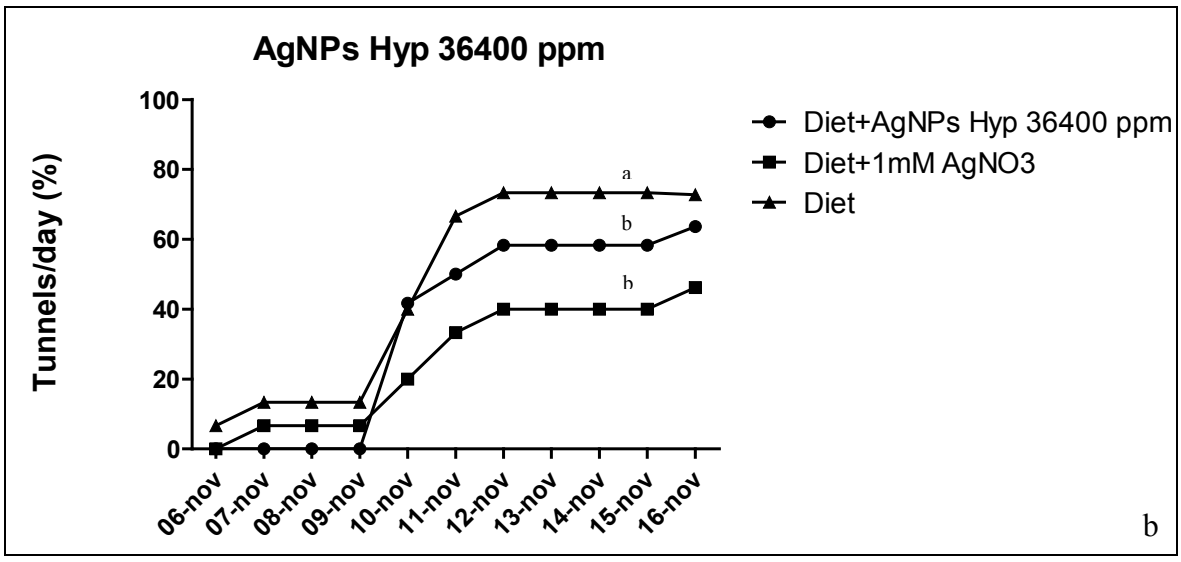


Figure 4.3. *H. obscurus* individuals that excavated tunnels/day (%) fed on artificial diet supplemented with silver nanoparticles from *H. perforatum* extracts (AgNPs Hyp) at 9200 and 36400 ppm. Controls were fed with artificial diet alone or with artificial diet supplemented with 1 mM $AgNO_3$. Different letters indicate significant differences between treatments based on Friedman test followed by a Dunn's Multiple Comparison Test ($P \leq 0.05$).

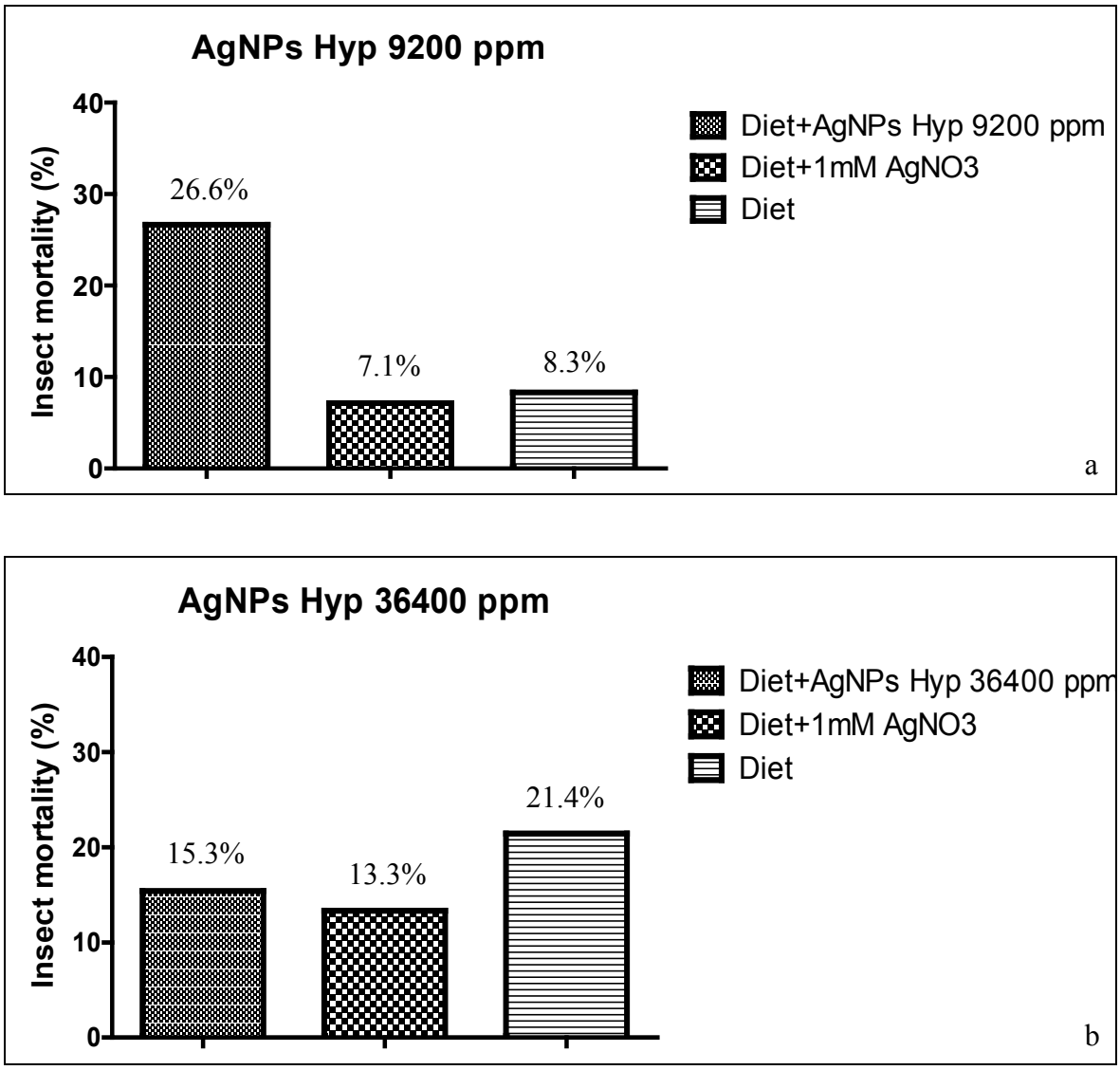


Figure 4.4. Mortality (%) of *H. obscurus* insects fed on artificial diet supplemented with silver nanoparticles from *H. perforatum* extracts (AgNPs Hyp) at 9200 and 36400 ppm. Controls were fed on artificial diet alone or with 1 mM $AgNO_3$.

4.5.3 Bioassays with nanoparticles produced with *G. officinalis* extracts

In feeding bioassay, the percentage of weight increase was statistically lower in individuals fed on diet supplemented with nanoparticles at 9200 ppm in respect control fed on diet with silver nitrate (Figure 4.5a); while no statistically differences in weight increase was observed among the different treatments at 36400 ppm (Figure 4.5b).

The percentage of tunnels excavated in the diet was the same between individuals fed on diet supplemented with nanoparticles at 9200 ppm and controls (Figure 4.6a). However, at the highest concentration (36400 ppm) the percentage of tunnels excavated in the diet was lower in individuals fed on diet supplemented with nanoparticles than the control fed with artificial diet alone, but no difference in the excavation behavior was observed in control insects fed with diet supplemented with silver nitrate (Figure 4.6b).

The mortality in insects fed on diet with nanoparticles at 9200 ppm was 11% on the total individuals treated, in adults fed on diet with silver nitrate was 20% and in insects fed on diet control it was 5% (Figure 4.7a).

The mortality of the insects fed on artificial diet with nanoparticles at 36400 ppm was 21% like the adults fed on the diet alone, while in adults fed on diet with silver nitrate was 15% (Figure 4.7b).

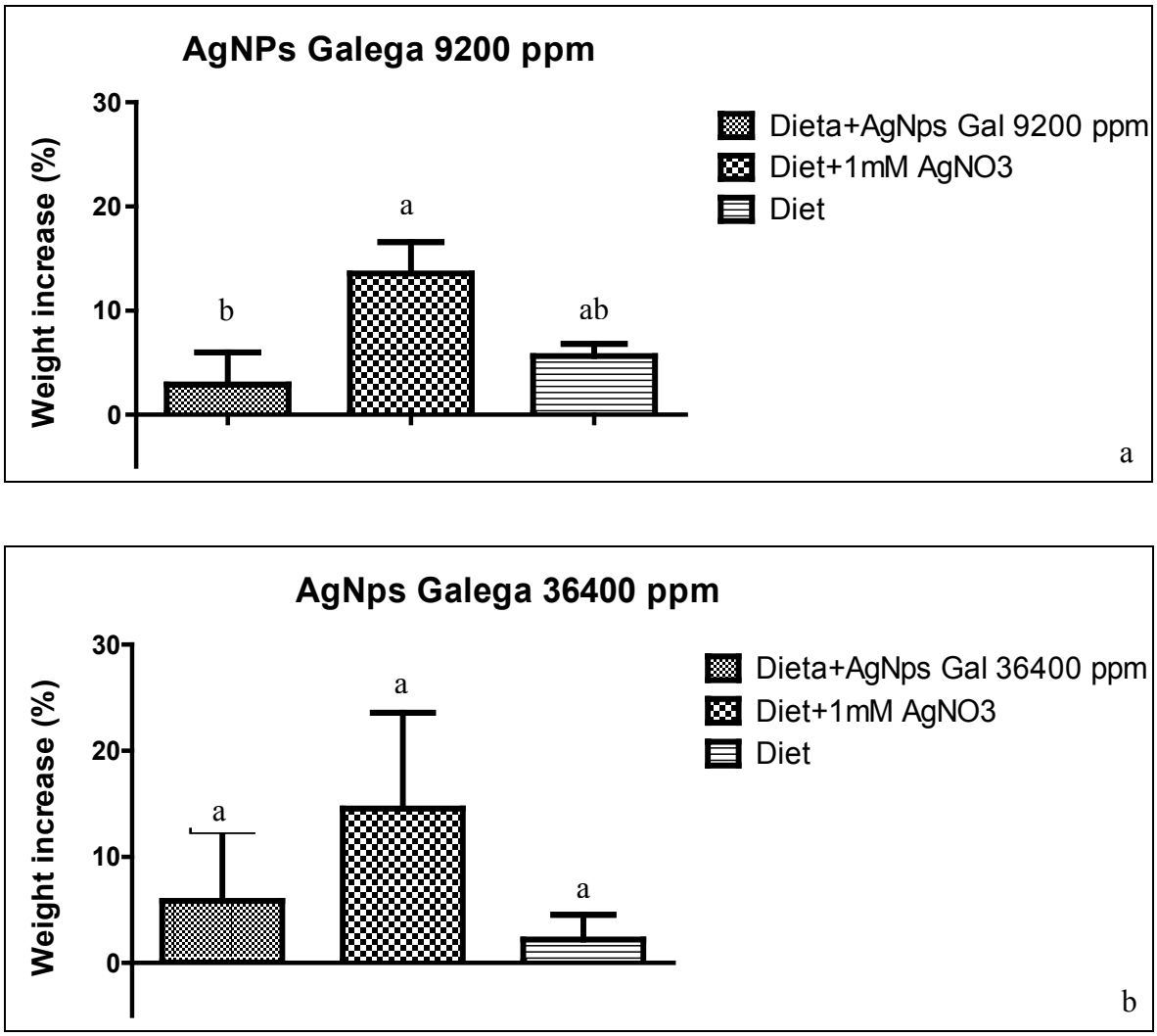


Figure 4.5. Weight increases (% ± SEM) of *H. obscurus* individuals fed on artificial diet supplemented with silver nanoparticles from *G. officinalis* extract (AgNPs Gal) at 9200 and 36400 ppm. Controls were fed with artificial diet alone or artificial diet supplemented with 1 mM $AgNO_3$. Different letters indicate significant differences between treatments based on a nonparametric Kruskal-Wallis test followed by a Dunn's Multiple Comparison Test ($P \leq 0.05$).

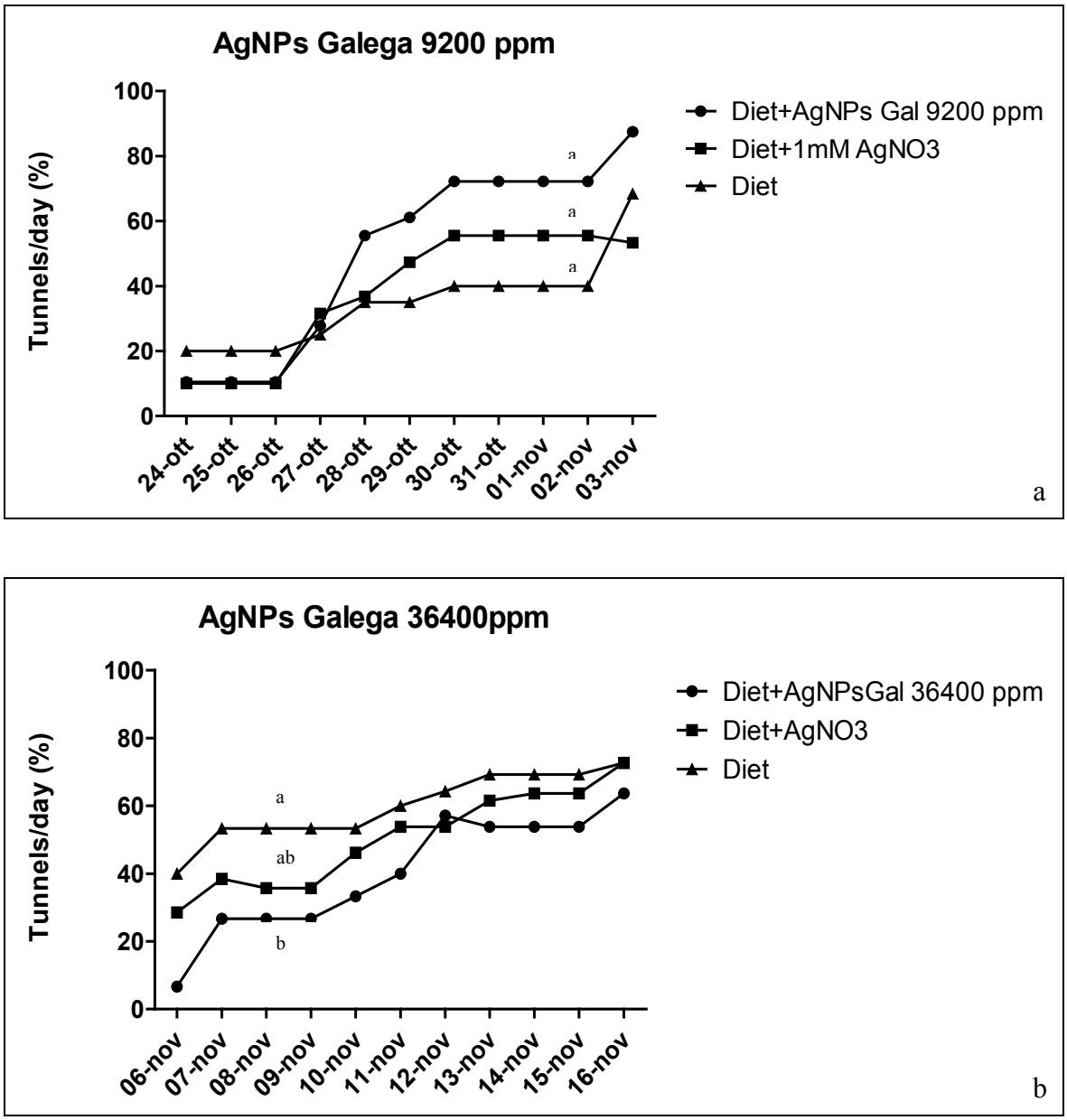


Figure 4.6. *H. obscurus* individuals that excavated tunnels/day (%) fed on artificial diet supplemented with silver nanoparticles from *G. officinalis* extracts (AgNPs Gal) at 9200 and 36400 ppm. Controls were fed with artificial diet alone or with artificial diet supplemented with 1 mM $AgNO_3$. Different letters indicate significant differences between treatments based on Friedman test followed by a Dunn's Multiple Comparison Test ($P \leq 0.05$).

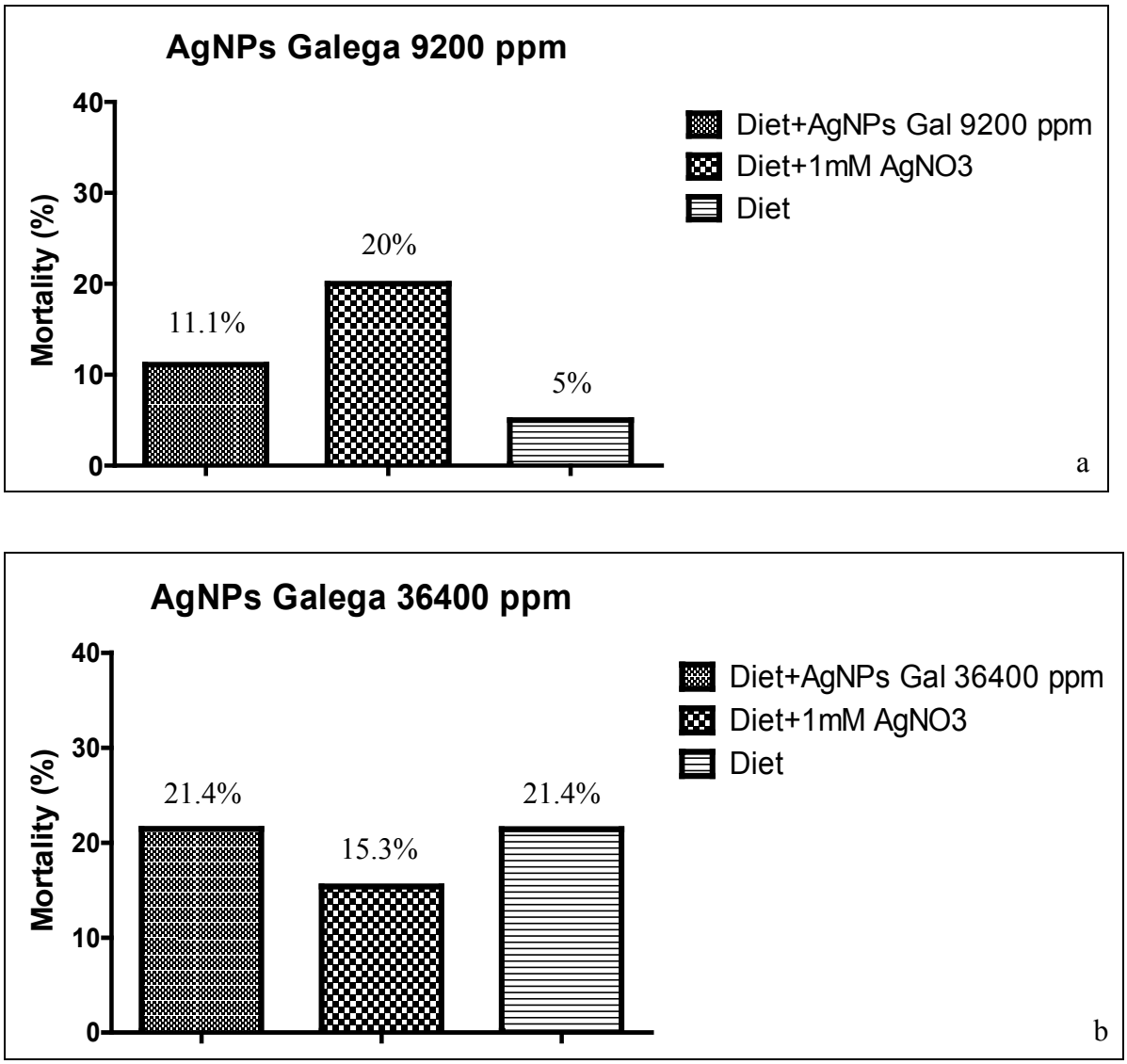


Figure 4.7. Mortality (%) of *H. obscurus* insects fed on artificial diet supplemented with silver nanoparticles from *G. officinalis* extracts (AgNPs Gal) at 9200 and 36400 ppm. Controls were fed on artificial diet alone or with 1 mM $AgNO_3$.

4.6 Discussion and conclusion

In the last decade, insecticidal activities of metal nanoparticles synthesized by different methods have been reported, but the use of nanomaterials in agriculture is still at a rudimentary phase. Several studies showed the effectiveness of nanoparticles in the control of human and crop insect pests.

Jayaseelan *et al*. (2011) showed the efficiency of silver nanoparticles synthesized using an aqueous leaf extract of *Tinospora cordifolia* against the

head louse *Pediculus humanus* and fourth instar larvae of the mosquitoes *Anopheles subpictus* and *Culex quinquefasciatus*.

In a study conducted on the tropical army worm, *S. litura*, the hydrophilic silica nanoparticles (SNPs) killed all the larvae within 24 hours after the treatment. *In vitro* toxicity of SNPs was evaluated on human fibroblast cell line and acute oral toxicity study in mice revealed that similar to the amorphous silica particle, its nanosized form is also relatively non-toxic.

Unlike conventional insecticides, SNPs kills the insects by causing damage to their cuticular water barrier. Insects loose water through their damaged cuticle and die because of desiccation. As this nanocide is physically active, insects are unlikely to develop resistance against it (Debnath, 2011).

In another study, Ag nanoparticles showed insecticidal activity against 1 day old first instar nymphs of *Aphis nerii* (Hemiptera: Aphididae) by dipping infested leaves and the somministration of AgNPs at concentration of 700 mg/ml showed the highest mortality (Rouhani *et al*., 2012a).

Moreover, SNPs and AgNPs have an excellent potential as stored grain as well as seed protecting agent (Rouhani *et al*., 2012b): nanosilica cause a reduction in the density of *Callosobruchus maculatus* (Coleoptera: Bruchidae) adults and this mortality could be attributed to the impairment of the digestive tract, while nanosilver is more effective on larval stage although the precise mechanism has not been clarified. Hypotheses on the toxicity machanisms include generation of reactive oxygen species, oxidative stress, membrane disruption, protein unfolding, and/or inflammation processes (Bragg and Rannie, 1974; Feng *et al*., 2000; Samuel and Guggenbichler, 2004; Elchiguerra *et al*., 2005; Reddy *et al*., 2007; Meng *et al*., 2009; Donaldson *et al*., 2009).

Nanoparticles loaded with garlic essential oil are efficacious against the flour beetle *Tribolium castaneum* (Yang *et al*., 2009).

In the present study it was assessed a different response between the use of nanoparticles produced from *H. perforatum* and *G. officinalis* aqueous extracts.

In general, AgNPs from *H. perforatum* extract showed the higher effects at lower concentration (9.2 g/L), in terms of *H. obscurus* mortality (Figure 4.4 a) and decrease of feeding activity (Figure 4.3 a). In fact, insects fed on diet supplemented with nanoparticles, started to feed on the fourth day of the bioassay instead on first day as controls, confirming the hypothesis that nanoparticles act as a feeding deterrent.

On the contrary, the results of the bioassay with AgNPs from *G. officinalis* extract, showed a decrease of the feeding activity in insects fed on diet with NPs at higher concentration (36.4 g/L) (Figure 4.6 b); this result was not supported from significative differences in insect mortality and weight decrease at the same concentration of nanoparticles. Contradictory results have been also reported in the bioassay with nanoparticles from *G. officinalis* at lower concentration.

These preliminary results have shown some positive responses but other replicates are pivotal to depict a clear conclusion.

The biosynthesis of metal nanoparticles using plant extracts is an emerging area of nanotechnology. The use of plant extracts in this process is an example of a simple green method. However, to compete with nanoparticles obtained through physical and chemical methods, it is necessary to regulate these green productions of nanoparticle using plant extracts and to develop systems for keeping expenses in check during their synthesis. To date, continuous methods for the synthesis of nanoparticles have so far been used only in small-scale production.

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