UNIVERSITÀ DEGLI STUDI DELL'INSUBRIA
Department of Science and High Technology
Ph.D. School in Chemical and Environmental Sciences
XXXII Cycle

Parasite-host relationships in the biological control of insects: strategies of immunoevasion/immunosuppression and interference of temperature on the lethality of entomoparasites

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Anno Accademico 2018/2019

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Abstract

In the last years, a great expansion of many species of insects considered harmful to crops and human health was observed, one of the reasons for this global phenomenon has been attributed to changing climatic conditions. In addition to their spread, changes in environmental temperature also seem to affect the life cycle of many species. These changes would easily lead to an uncontrolled spread of phytophagous and vectors species, in different areas around the world, where the presence of a few enemies and natural competitors would ease their distribution. Current integrated pest management practices suggest that any action to control the increased spread of vectors or phytophagous should not increase the use of synthetic chemical pesticides due to their well-known adverse effects. Therefore, eco-friendly methods based on the use of bio-insecticides such as insects and predatory mites, parasitoids, parasites and microbial pathogens should be intensified. Among bio-insecticides, most used are entomopathogenic nematodes (EPNs) and *Bacillus thuringiensis* (Bt).

Two aspects of the relationship between bio-insecticide and insect hosts were considered in this PhD project: the first has been the study the mechanisms carried out by EPNs complexes (*Steinernema carpocapsae* - *Xenorhabdus nematophila*) to overcome or neutralize the immune system of the insect host. The success of the EPNs results mainly from immunological disabling induced by nematode and its symbionts by immune evasion or immune depression process when released inside the host hemocoel. In this contest, the functions of structures and molecular components of the surface of both nematodes and their symbiont bacteria play a key role and, for this reason, we assessed the role of protein pools isolated from the surface of *S. carpocapsae* and from *X. nematophila* against *Galleria mellonella* (Lepidoptera). We carried out treatments with a high salt concentration to isolate surface proteins of both nematode and bacterium; the eluted compounds from live *S. carpocapsae* possessed a slight cytotoxicity on the haemocytes, whereas those from live *X. nematophila* markedly affected the host cells' viability. Bacterial proteins can inhibit the phagocytic activity, despite they strongly trigger the host prophenoloxidase-phenoloxidase system. The data obtained show that EPNs surface compounds play a key role in immunoevasion/depression of insect hosts, causing a severe physiological disorder.

The second part of the project is aimed to acquire useful information on host-parasite relations in the context of climate change and in particular, we reconsidered the physical conditions (temperature, humidity, etc.) in which bioinsecticides are highly effective. We evaluated the effectiveness of different commercial bio-insecticides (*Steinernema feltiae*, *Steinernema carpocapsae*, *Heterorhabditis bacteriophora* and *Bacillus thuringiensis*) assessing the mortality rate induced in two insect models, Galleria mellonella (Lepidoptera) and Sarcophaga africa (Diptera) after conditioning at various temperatures (10, 20 and 30 °C); moreover, we investigated the effects of temperature on the basal humoral immunity (phenoloxidase and lysozyme activity).

The results show that *G. mellonella* is susceptible almost to all bio-insecticides at all the examined temperatures, whereas *S. africa* is more susceptible at 30 °C to all bioinsecticides. Temperature can modulate PO activity of both insect models, even if variations in lysozyme activity is observed only in *G. mellonella*. Our data suggest that temperature changes can alter the relationships between entomopathogens and their hosts, thus affecting the efficacy of the bio-insecticides on the target insects in specific ways in different species. Temperature can also influence the host basal immune response even if, in some instances, the enhancement of immune activity does not seem to be the main factor responsible for an increased resistance to entomopathogens. Therefore, the balance between the success of a bio-insecticide and the insect survival can differ as a result of environmental variations, which could be responsible for physiological changes of both the host and the entomopathogen.

In the last phase of the PhD project aimed at the control of harmful insects, the efficacy of the entomopathogenic nematode, *S. carpocapsae* and its symbiont bacterium was evaluated for the control of the spotted wings Drosophila, *Drosophila suzukii*. In recent years, the serious damage caused by *D. suzukii* to thin-skinned fruit, have led to the need to study effective systems for its control and its undisturbed spread. The greatest difficulty in protecting crops from fruit flies is the fact that it affects the fruit during ripening, when it is not possible to carry out unsafe treatments due to imminent harvesting. To date, knowledge about the behavior and the fight against this midge is still inadequate, considering the relatively recent spread on our territory. Our preliminary data suggest that the nematode and its symbionts interfere with some fundamental

processes (proPO system, phagocytosis, cell encapsulation) of the immune system of *D. suzukii* by means of immunoevasion and immunodepression strategies.

We believe that data obtained in this project can contribute to increasing knowledge of insect host–parasite relationships will greatly improve our understanding of non-self-recognition processes that are typical of insect immunity and could be a good starting point for improving the biological control of insect species potentially harmful to agriculture, urban greenery and perhaps animal and human health.

CHAPTER 1

Introduction

During 300 million years, insects have been the only living organisms who have colonized every possible ecological niche and that have used all possible sources of food. In the course of their evolution, each group or species acquired many peculiar characteristics that made it well adapted to that environment. The multifaceted features of insect's species also deeply influenced the evolution of plants and animals associated with them (Farb, 1962).

The result of this highly diffusion and diversification (fig.1) in terms of species (the classified species are about 1 million, while it is thought that the unknown species could be 5-10 million) (Stork, 2018) is that still today more than half of the species have not been discovered, giving priority to those that have an economic importance for humans (Grimaldi et al, 2005).

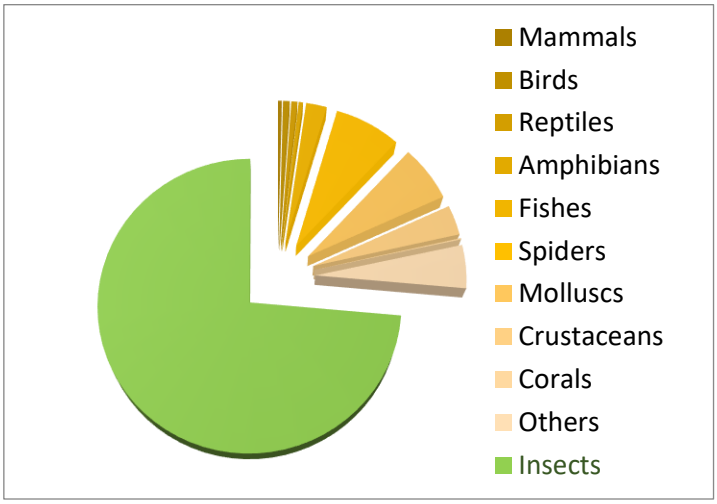


Fig.1 - Distribution of animal species on Earth.

Among insects, there are social behaviors which are characterized by a high organization; some of them are very simple and primitive, others highly organized; moreover, they differ in morphology and many physiological factors, such as, development, resistance to infections and environmental changes, immune response and reproduction.

There is no place on Earth where there are no insects, for example about forty species live in Antarctica; not only plants and flowers, but also beetles, moths and butterflies have been found beyond the Arctic Circle, as far as plants and flowers grow, and stinging insects can be found at any latitude up to where there are warm-blooded animals they feed on (Farb, 1962).

Morphologically, insects also have the largest variety of sizes: the smallest insects are smaller than some protozoa, the largest can also get to be larger than some small mammals such as mice and shrews. Millions of years ago could have been even bigger: the fossil of a found

dragonfly shows a wingspan of 75 cm. Most insects, however, are not longer than 0.5 cm and this avoids competition with larger animals.

Insects have segmented bodies, articulated legs and external skeletons (exoskeletons). They differ from other arthropods by their body, which is divided into three main regions: the head (which carries the mouthparts, the eyes and a pair of antennas) the thorax (with three segments that usually has three pairs of legs in adults and usually one or two pairs of wings) and finally the abdomen with several segments, which contains the digestive, excretory and reproductive organs (Chinery, 2010).

The Order of Insects with the greatest variety is represented by the Coleoptera, of which today we know more than 350,000 species. Among the advantages of this extraordinary success we have surely the complete metamorphosis they undergo, passing from a larval stage to the winged form as an adult, then the outer shell that protects them effectively, and finally the enormous differentiation of the mouthparts that have been able to adapt to almost any type of feeding.

The second order in terms of number of species known today (over 160,000) is the Lepidoptera, whose differentiation develops in the beautiful shapes and colors of butterflies and moths. The wings, one of the main characteristics for the recognition of lepidoptera, are composed of scales (hence the scientific name) and can also be large compared to the body. The distinction between butterflies and moths consists mainly in the position of the resting wings (open for moths), the habit of flying at dusk of moths and the reverse direction with which the butterflies spin the cocoon.

The Hymenoptera are another well-known Order of insects, whose species discovered are over 120,000. This order includes the well-known wasps, bees and ants. One of the main characteristics of these insects is their organization in society, which makes them almost unique in the range of invertebrates. The role often played by the components of a society is associated with the concept of "caste", in which every individual carries out his or her work in the exclusive interest of the colony's well-being. Still debated today is the instrument that is often used to regulate this complex organization, even though it is widely accepted the use of pheromones by some individuals to regulate the various activities of the colony.

Finally, another well-known order is certainly the Diptera (over 100.000 species discovered) to which essentially belong flies, horseflies and mosquitoes. This order belongs many of the

species harmful to humans and animals. They can often become important vectors of diseases such as malaria, sleeping sickness, filariasis and yellow fever, having developed, among other things, the specific characteristic of sucking blood from their victims (Gullan et al, 2014).

1.1 The evolutionary success of insects

The success of insects is mainly due to characteristics such as the flight, the adaptability, a protective exoskeleton, the small dimensions, a fast reproduction and the metamorphosis process (Farb, 1962).

1.1.1 Flight

Thanks to their wings, insects were able to spread all over the planet; indeed, when the living conditions in one place became disadvantageous, they moved to another place, colonizing it. The possibility of having wings has also given them great advantages, for example in the search for partners, food and in the escape from possible dangers.

1.1.2 Adaptability

In addition, no other form of animal life has been able to adapt like insects to such different living conditions, as is shown, for example, by the enormous variety that we find in different alimentary habits. As well as feeding on all types of higher plants, some insects can feed on synthetic material and other substances without any nutritional value. Alongside the variety in the diet, there has also been a considerable differentiation in the dedicated mouth and chewing structures. What, for example, in the primitive insects were articulated appendages, then became differentiated structures such as: the butterfly suctorial proboscis (ideal way to suck the nectar from the flowers), a tool to puncture the skin and suck the blood in mosquitoes, a pincer in beetles or a kind of hypodermic syringe in aphids.

1.1.3 Defensive features of the exoskeleton

Regards the external skeleton (exoskeleton), this is cylindrical in shape, that allows the greatest construction strength with minimal use of material. The external skeleton is formed by the hardening of secretions produced by the insect and is a remarkable protective shell. Its main component is chitin, a very flexible, light and resistant polymer. The rigid segments are further

reinforced by substances such as cuticle, which is similar in composition to human nails (Noh et al, 2016). Finally, the entire exoskeleton is covered with waxes that give to the insect both the impermeability to external water and protection from dehydration. A further important characteristic of the exoskeleton is the great variety of body structures such as jaws, spines, protections, stings, etc (Parle et al, 2017).

1.1.4 Dimensions

The fourth reason for success is the small size of the insects. Small organisms have indeed minimal requirements related to the environmental demand: a very small quantity of food can represent a whole meal for an insect, as well as a drop of water can satisfy its entire necessity. Another reason for the winning strategy of small size is obviously that the excessive size of the body can lead to limitations, one of these is the strength of the exoskeleton: as it grows it becomes progressively weaker. Finally, as we know, insects can breathe by means of an intricate and microscopic system of tracheas and tracheoles that carry on oxygen to any part of the body; the air penetrates these tubes by simple diffusion of the gas molecules, a method that obviously works if the size and distance are minimal. Indeed, the largest species of insects are found in warm tropical regions, because gases spread more rapidly at high temperatures.

1.1.5 Reproduction

Reproductive success is one of the most significant measures of an organism fitness. In insect populations, females often produce large numbers of eggs (high fecundity), most of the eggs hatch (high fertility), and the life cycle is relatively short (often as little as 2-4 weeks). Together, these three characteristics enable insects to produce remarkably large numbers of offspring. A typical female lays 100-500 eggs in her lifetime, but numbers in the thousands are not uncommon. The queen of an African termite colony may be the mother of more than ten million workers during her 20-25 years lifespan. This could partially explain how Insects evolve at a high rate and adapt quickly to changing environmental conditions.

1.1.6 Metamorphosis

Finally, regard the metamorphosis, it consists of a physiological change through three main stages leading from the caterpillar to the butterfly or from the larva to a beetle. The difference between the larval stage and the adult insect is indeed substantial: the larva can eat a certain

amount of food, while the winged adult insect feeds on completely different food or in some cases they don't eat. Unlike higher animals, where only one fifth of life is spent to reach adulthood, the insects spend most of its life as a larva and during this time makes a significant amount of adaptations to survive (Bland et al, 2010).

1.2 The ecological role of insects: beneficial and harmful insects.

The concept of "beneficial" and "harmful" is very often related to an exclusively subjective human judgment. Indeed, we identify as beneficial the species that provide help or that are economically exploitable and from which we therefore have a return in economic terms. In the same way, the harmful species is that which creates damage in economic terms for humans and for urban green or which, more significantly, can be a vector of disease for both humans and animals. Hence the need for the study of insects from an immune point of view comes in: both for the protection of useful species, and for the contrast of those defined as harmful to man.

1.2.1 Beneficial species

Among the insects, the common bee (*Apis mellifera*) is probably one of the most studied insects in the world and certainly also the most important pollinator insect. The complexity of the social structure in which the hive is divided and which provides for the existence of 3 castes has also been studied a lot: the queen, the only one able to reproduce, laying up to 1000 eggs a day; the males, called drones, who are present only in spring in a few hundred individuals and have the exclusive role of fertilizing eggs; the workers, sterile females who perform all the tasks necessary for the health of the hive, such as collecting nectar and pollen, clean the cells, feed the larvae (with honey) and the queen (with royal jelly), defend the community from the attack of predators and parasites (Nouvian et al, 2016).

Among the species of insects useful to man there is the silkworm (*Bombix mori*). Its use to produce silk has been known for thousands of years and it is native to the Asian continent, feeding exclusively on mulberry leaves. It belongs to the Order of Lepidoptera, at the adult stage is therefore a moth, although its usefulness in economic terms plays at the larval stage and when it then pupates forming the cocoon of silk. This is simply a polymer produced directly by the salivary glands of the silkworm that solidifies in contact with air, and, due to the "eight" movements the

animal makes with the head, it creates the layers that make up the raw silk cocoon (Tabunoki et al, 2016).

The cocoon is made up of a single continuous string, the length of which ranges from 300 to 900 meters. The silkworm takes 3-4 days to prepare its cocoon and layer after layer it goes on producing 20-30 concentric levels made of a single thread. At the end of this weaving work, it turns into a chrysalis and then flicker. When the metamorphosis of the caterpillar ends, the adult insect leaves the cocoon by making a hole and secreting a substance that ruins the wire. For this reason, the breeders must kill the chrysalises by subjecting the cocoons to a drying process in special machines. Alternatively, the death of the caterpillar is caused by immersion in boiling water.

Some cocoons are stored to allow the insect to reproduce. The life of moths, however, is very short because they are unable to fly or feed. The reason for this is that the silkworm is the result of a long genetic selection practiced by man, which over the centuries has lost many of the original characteristics, including the ability to fly or mimicry (Herold et al, 2017).

Another example is the black soldier fly (*Hermetia illucens*), that is a native Diptera of the American continent whose larvae are frequently found in composting and waste disposal plants, where they play a useful role in reducing the mass and pollution load of waste itself. The extreme voracity and the rapid development cycle of *H. illucens* larvae can therefore be exploited for the disposal of organic waste with a high environmental impact, such as slurry from intensive livestock farms, waste from the agro-food industry and solid urban waste. In recent years, waste and solid waste disposal and recycling systems have been developed based on biodigestors and composting plants that use insect farms to reduce the volume of waste at relatively low costs.

The benefits of this use range from reducing the volume of waste, estimated at an average value of 30-55% in two weeks, to reducing the pollution load of the waste itself and the lower health impact compared to other digestion systems (Wang et al, 2017; De Smet et al, 2018).

1.2.2 Harmful species

One of the main dangers when it comes to harmful insects is represented by the vectors of diseases. Indeed, there are many places on Earth where there are conditions in which many species of harmful insects can grow and reproduce under optimal conditions. The diseases they

can bring to humans can be both direct (through skin puncture), but also indirectly, infecting the animals that man feeds on (Dobson, 2004).

A further important problem that harmful insects can cause is the damage of crops and foodstuffs. Every year, indeed, we hear more and more about the invasions of alien phytophagous species that do not find natural competitors, and that can therefore freely reproduce and grow to the detriment of agriculture and crops.

In recent years, the crops of many fruit plants species are threatened by the "spotted wing fly" *(Drosophila suzukii*, Diptera, Drosophilidae) originating in Southeast Asia. This small insect has recently spread, probably brought with goods from the East. It has found a favorable environment in Europe and has proliferated at the expense of small fruit crops. The fly causes serious and irreversible damage to several species of thin-skinned fruit. *D. suzukii* is spreading undisturbed, in the absence of effective natural antagonists. It is not easy to protect the crops from this fruit fly, as it hits the fruit during its maturation; in this phase, indeed, it is not possible to carry out treatments due to the imminent harvest. The speed of reproduction also makes the insect particularly dangerous for orchards and vineyards: the females can reach 10 generations in a year (Asplen et al, 2015).

Another important phytophagous insect that has become widespread in recent years is the Asian bug (*Halyomorpha halys),* as the name suggests, its origins are oriental: it comes from China, Japan and Korea. It was first reported in Europe in 2007, in 2010 it reached the United States and in 2012 it also landed in Italy. *H. halys* can cause considerable economic damage to agriculture: the Asian bug is ravenous and multiplies quickly. Given the absence of natural predators, the Asian bug has a free field and can cause severe damage to crop in the garden or on the farm. Its life cycle is annual but in Asia, on its natural habitat, the Asian bug can make 4-6 generations per year. Adults are present in the crops in spring and summer, while they spend the winter sheltered, in adult form, hidden under the barks of trees, shrubs, under the stones or among the dried-up vegetation. In favorable conditions, during the winter period, the Asian bug can take shelter also in the house (Leskey et al, 2017).

Over the years, therefore, different strategies have been developed to control insect vectors and pests.

1.3 Pests control methods

The strategy to contrast species harmful to humans and the environment often includes the use of chemical insecticides (pesticides) (Kim et al, 2017). These can be classified in various ways, depending on whether their way of action or their chemical structure is considered. The classification based on the mode of action classifies the insecticides into poisons by ingestion, contact and acting by the respiratory tract. Insecticides by ingestion act by penetrating insects orally, insecticides by contact by penetrating the body of the insect through the tegument and, finally, there are pesticides whose action takes place in the form of gas or steam by penetrating the body of the insect through the spiracles of the trachea. Each of the main classes can also be further subdivided into sub-classes based on the level of toxicity and other properties (tab.1).

Category	Activity time	Compund class
not persistent	1 - 12 weeks	Phosphorganic
moderately persistent	1 - 18 months	Carbamate
persistent	2 - 5 years	Chlorogenics
permanents	permanent	Hg, As, Pb-containing

Tab. 1 - Classification of chemical pesticides.

However, the uncontrolled use of chemical pesticides with a broad spectrum of action, with considerable persistence and high acute toxicity, has subsequently highlighted the negative aspects of the use of these substances in crops, especially in the long term. One of the main problems is the accumulation of residues at the top of the food chain, but also the ecological disruption due to pollution of groundwater and waterways, increased production costs and increased risks to public health (Roy et al, 2017).

1.3.1 Possible use of bio-insecticides: the biological control

In recent years there has been the necessity to introduce substances that have both efficacy in the elimination of harmful species, but harmless for the environment: the use of bio-insecticides (or insecticides from natural origin) is therefore increasingly used. Bio-insecticides are based on bacteria, fungi, oomycetes or viruses that can be used to control pests or diseases vectors and that are used in the wider process of biological control.

Biological control is indeed a technique that exploits the antagonism relationships between living organisms to contain the populations of the harmful ones. This technique has evolved to safeguard agriculture and it is generally applied for the protection of crops and foodstuffs, but it

can also be applied in any context that requires the control of the population of a certain organism that is harmful to humans, animals or the environment.

Biological control can be carried out with different alternative strategies. One of the most widely used methods is the introduction of one or more natural enemies of the organism that we want to contrast by allowing natural regulation in the population (Hajek et al, 2007). Obviously, this is a completely different method to the use of chemical pesticides, particularly in terms of responsiveness: the use of pesticides is certainly faster, but harmful to the environment, animals and people.

A microorganism that is widely used to fight harmful species (especially phytophagous insects) is the bacterium *Bacillus thuringiensis (Bt)*. *Bt* is naturally present in the soil and it is not toxic to humans, plants or animals. Its action is carried out through the production of spore and a crystal protein with insecticidal action. The larvae of the insects that ingest the spores and crystals of *Bt* suffer intestinal damage, intestinal lesions are due to the solubilization of the crystals that turn into toxins (*Cry e Cyt*). Toxins, in the intestine of the larva, bind to receptors and lead to the perforation of the wall and therefore to the death of the larva (Cossentine et al, 2016). The insecticide action occurs by ingestion and not by contact as with classic insecticides. Moreover, the efficacy of *Bt* is limited to the larval stage of insects. In other words, it is not an insecticide that destroys eggs or affects adults; it only works in a targeted manner on larvae, preferably in the early stages of development. The mechanism of action is therefore highly specific and selective, based on the binomial toxin-receptor interaction.

Therefore, the *Bt* is an effective insecticide only against certain species of insects. This means that it is totally harmless to useful insects in the garden, such as pollinators (bees and bumblebees) and predatory insects. The high rate of degradation of spores and crystals in the sun's rays means that their presence in the environment is really reduced. Moreover, it is totally harmless for birds, fish and, of course, for man. In addition, a problem in the application of *Bt* is the leaching on the leaves that takes place in unfavorable weather conditions (fig. 2).

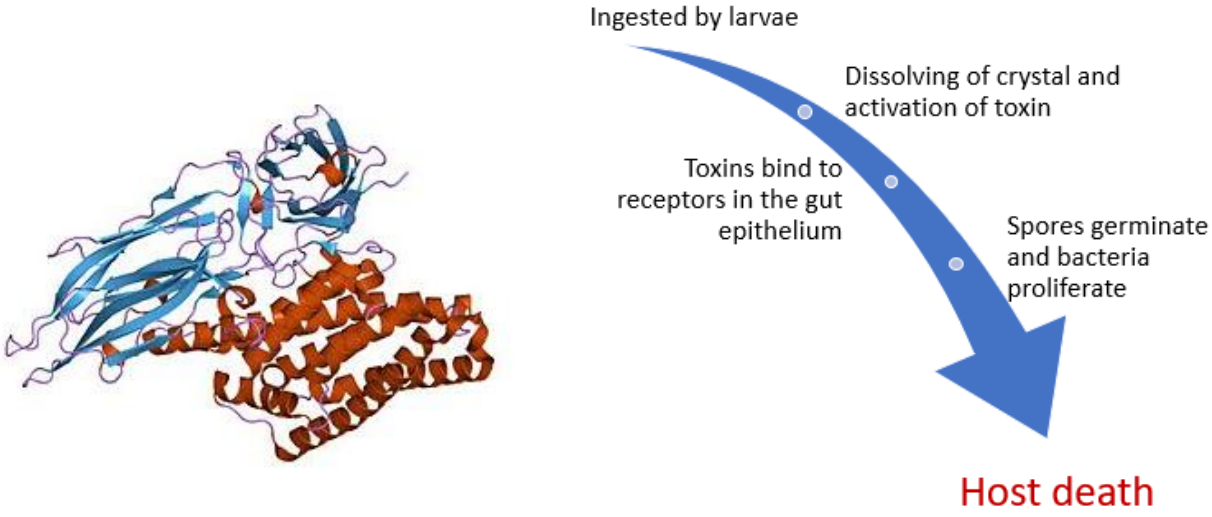


Fig. 2 - At left: the Bt Cry toxin, at right: action mechanism of Bacillus thuringiensis

A second effective bio-control agent is the fungus *Beauveria bassiana.* The fungus causes damage to different types of insects, acting as a parasite; when the fungus spores reach an insect target, they enter the body, germinate, develop and kill the insect using it as a source of food. After the death of the insect, the mycelium develops on the body, producing new spores (fig. 3). The insect, parasitized even if not yet dead, spreads the fungus to other specimens during its movements. The proliferation of material from the fungus will be followed by the production of toxins that will cause the parasite death within 3-5 days (Bruck, 2009).

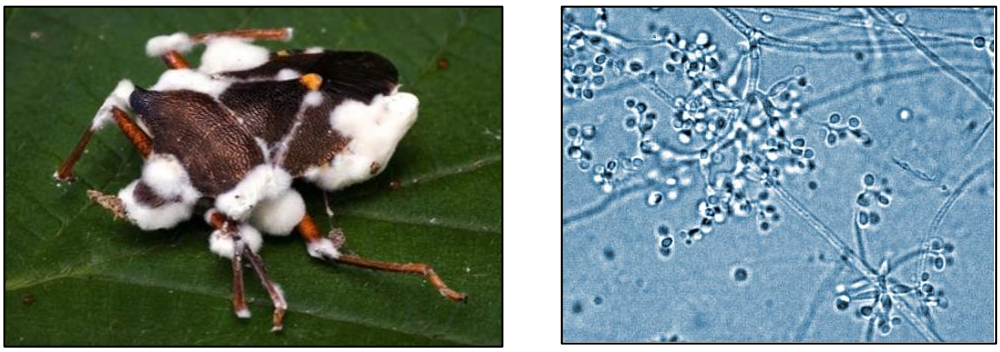


Fig. 3 - Action of B. bassiana against insects. At right, spores of B. bassiana.

Particularly effective and widely used in biological control are the entomopathogenic nematodes (EPNs). Nematodes are one of the most abundant groups of animals on earth (Blaxter et al, 2003), and due to their small size, resistant cuticle and ability to adapt to rapid environmental changes, they have colonized a wide range of habitats including bodies of

vertebrates and invertebrates, so they can be defined as free-living or as parasites (Blaxter et al, 1998; Schmid-Hempel, 2008).

A small but significant group of parasitic nematodes, called entomopathogenic nematodes (EPNs), is very interesting because its members possess a number of features that allow them to be used as a biological control agent against insect pests (Gaugler et al, 1990; Georgis et al, 1994; Poinar, 1998). To EPNs belong the families *Steinernematidae* and *Heterorhabditidae* (Nematoda, Rhabditidae). All the members of these families live in a specific mutualistic symbiosis with bacteria: *Xenorhabdus*, associated with *Steinernematidae* (Poinar, 1979), and *Photorhabdus*, associated with *Heterorhabditidae* (Forst et al, 1996; Forst et al, 1997; Forst et al, 2001), both nematodes and symbiotic bacteria cooperate to kill the host target.

The nematode acts as a vector for symbiont bacteria and, by a rapid modulation of the response of the host immune system, it prepares a suitable environment for its symbiont (fig. 4, short-term phase), which after the release, proliferate and establish suitable conditions for the reproduction of the nematodes, providing nutrients and inhibiting the growth of other

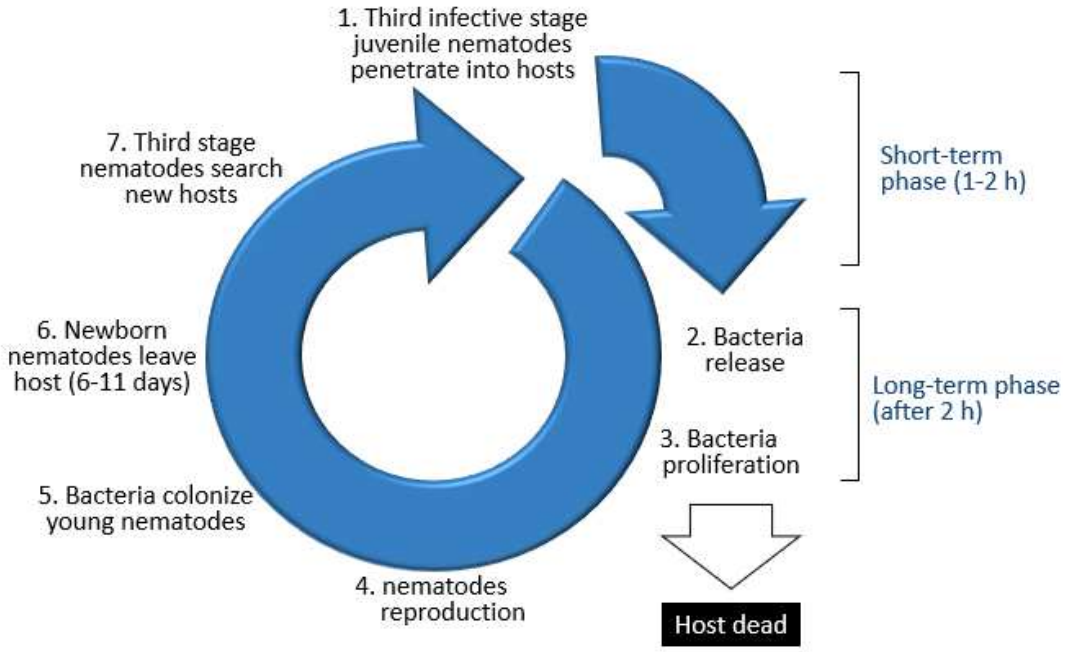


Fig. 4 - EPN: nematode-symbionts life cycle and effects on the host

microorganisms on the host corpse by means of the release of antimicrobial compounds (fig. 4, long-term phase).

The relationship between nematode and its symbionts is essential for the efficiency of biocontrol and allows nematodes to be exploited against a wide range of harmful insects (Dunphy et al, 1990; Eleftherianos, 2018).

1.4 Evasion/depression strategies: how parasites interfere with the insect immune system

As mentioned above EPNs are complexes of nematodes-bacteria (nematocomplexes) and the success of their strategies is based on the synergy between the parasite itself and its symbiotic bacteria. In general, endoparasites penetrate insects by crossing the first line of defense, consisting essentially in the exoskeleton and once they reach the hemolymph in the haemocoelic cavity (i.e. the open circulatory system of insects), they must evade the host recognition system or depress the immune processes (Schmid-Hempel, 2009) (fig. 5).

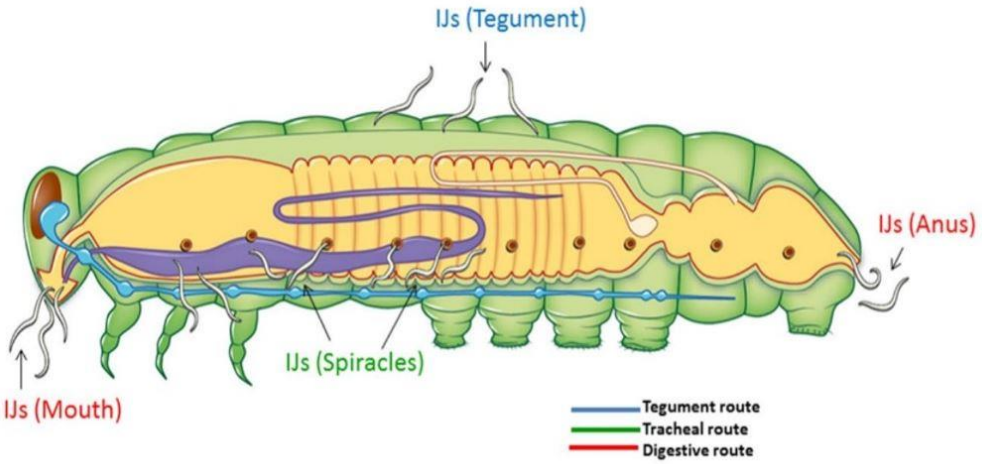


Fig. 5 – Scheme of infection and immune challenge by EPNs.

The two main strategies by which EPNs avoid and neutralize host immune defenses are the molecular disguise/mimicry and immunosuppression processes. These strategies can be particularly effective when parasites penetrate young larvae with a low level of immune response. However, EPNs are able to invade both adults and dead insects (Griffin et al, 2012; Castillo et al, 2012). In general, molecular mimicry processes can be achieved by the synthesis of molecules that are antigenically related to the host (usually called self-proteins) and that are exposed on the surface of the parasite body (Bayne et al, 1987; Damian, 1991).

An interesting example is the strategy used by the parasite *Steinernema feltiae* to infects its targets insects. The most important characteristic of this EPN is its ability to elude the host immune surveillance which allows it to live in the hemocoelic cavity undisturbed. To evade the host recognition the worm exploits the mimetic properties of its body surface (cuticle/epicuticle) (Politz et al, 1992; Akhurst et al, 1993; Brivio et. al. 2008). During the infection the outer epicuticular layer can be changed both in composition and organization; these variations are related to the host body environment, and every time the EPNs moult, they change their body surface building a different cuticle. Cuticles of many entomopathogens nematodes, together with

other surface molecules and secretions, cooperate in immune evasion and suppression of host defenses (Cox et al, 1981).

Steinernema carpocapsae is also effective to neutralize the host immune response, even if its cuticle seems not be directly involved in immunoevasion. As mentioned above, the action of the nematode itself occurs in the early stages of infection but, after a variable time (1-2 hours), the nematode release, from specific vesicles in the gut, its symbiont bacteria into the circulatory fluid of the host. The action of bacteria, combined with the immuno-depressive processes induced by the nematode, culminates in the death of the host due to septicaemia, sepsis and *blood poisoning*[*].

Many studies have described the physiological disturbance of the host caused by the release, proliferation and production of toxins by symbiotic bacteria (Chattopadhyay et al, 2004); these microorganisms seem to reorganize the environment (the host body) in such a way as to promote their survival and reproduction of the parasite. In particular, *Xenorhabdus* owes its effective virulence to the presence of various surface structures such as pilin/fimbriae, flagella and the presence and release of external membrane vesicles (Khandelwal et al, 2003; Ellis et al, 2010). These structures interact with the host and influence its recognition by the blood cells; they also prevent the processes of phagocytosis and nodulation (pili/fimbriae), promote adhesion and invasion in the host tissues (flagella) and release proteases, thus contributing to larvicidal activity (Brivio et al, 2018). The lethal action of symbiont bacteria is therefore obtained through the immunoevasive/depressive and toxic action of both external structures and secondary

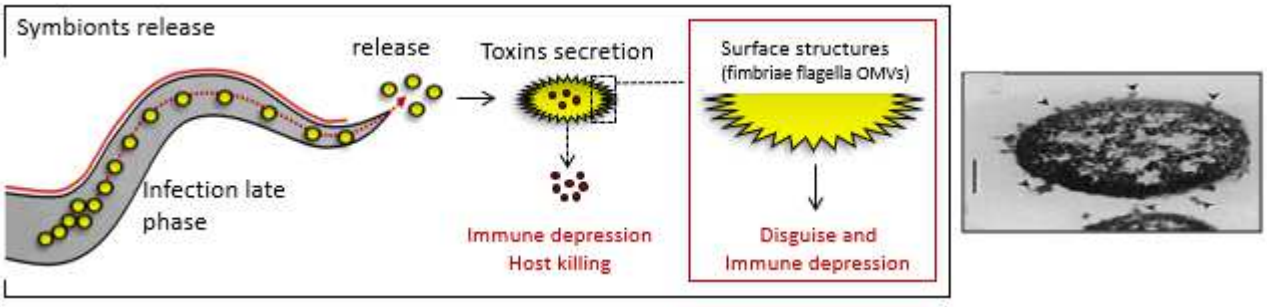


Fig. 6 - The action of EPNs and the external structures of bacteria symbionts (photo at right).

metabolites secreted by the bacteria; the overall action of these toxic components causes a severe metabolic and functional disorder that leads to the host death (fig. 6).

[*] Septicemia is defined as having bacteria in the bloodstream that cause sepsis. Some people call septicemia "blood poisoning," and this term is accurate since the overwhelming bacterial infection can indeed poison your blood. Sepsis is defined as an extreme inflammatory response to infection. When your body is threatened with a severe infection your immune system responds by releasing chemical messengers to sound the alarm. These chemical messengers produce inflammation throughout your body. The infection can be due to bacteria in the bloodstream, but sepsis can also be produced by an infection that is present only in one part of the body.

CHAPTER 2

Insects' immunity

2.1 Parasite-host relationships at molecular level

Because of the spread of insect species that occupy almost all the Earth's habitats (reflecting the great success of this group), many insects live in environmental conditions full of parasites or pathogens. Insects can than survive mainly thanks to the extreme effectiveness of their immune system: any parasite must therefore interact and win against these defenses to survive. Many parasites, indeed, survive and reproduce in invertebrate hosts that have an immune system designed to remain intact and to discriminate themselves (*self*) from others (*not self*) (Brivio et al, 2005).

Invertebrates do not have immunorecognition receptors but instead possess useful pattern recognition receptors (PRRs): these factors can interact with a wide range of foreign antigenic compounds (called PAMPs and defined as Pathogens Associated Molecular Patterns). PAMPs-PRRs interaction is a key process of innate immunity that triggers the mechanisms based on the effectors responsible for the elimination of non-self (Medzhitov, 2001). PAMPs are compounds (including oligosaccharides, proteins, glycoproteins, lipids) that are essential to the survival of microorganisms. An important characteristic of PAMPs is their preserved structure, which is the same among the organisms of a class (Janeway and Medzhitov, 2002).

2.2 The immune system of insects

In order to better understand the scientific-experimental rationale of the project, it is necessary to describe the essential characteristics of the immune response of insects. Understanding how insects defend themselves against parasites and bacteria is extremely useful to a better comprehension of the evasive/depressive strategies implemented by EPNs.

Investigation of insect immune system can be performed by experimentally separating the umoral and cellular defenses. The humoral responses are characterized by soluble molecules such as antimicrobial peptides (AMPs) and the enzymatic cascades (prophenoloxidase-phenoloxidase

system) leading to melanin formation. In insects, the blood and interstitial fluid are identical and are referred as hemolymph in general, which wets all internal tissues, organs or hemocytes, and facilitates the transport of nutrients, waste products and metabolites in general. The cellular defenses, in contrast, are characterized by hemocytes-mediated immune responses like the phagocytosis, the nodule formation and encapsulation processes (Nappi et al, 2004).

2.3 Humoral defenses

The main processes among humoral responses of the insect immune system, are melanization (proPO system activity) and antimicrobial peptides (AMPs) synthesis (Kavanagh and Reeves, 2004).

2.3.1 proPO system

One of the most important humoral process against a wide range of pluricellular pathogens is the melanization, also responsible for wound healing as well as nodule and capsule formation. The formation of melanin is catalyzed by phenoloxidase-monophenyl L-dopa: oxygen

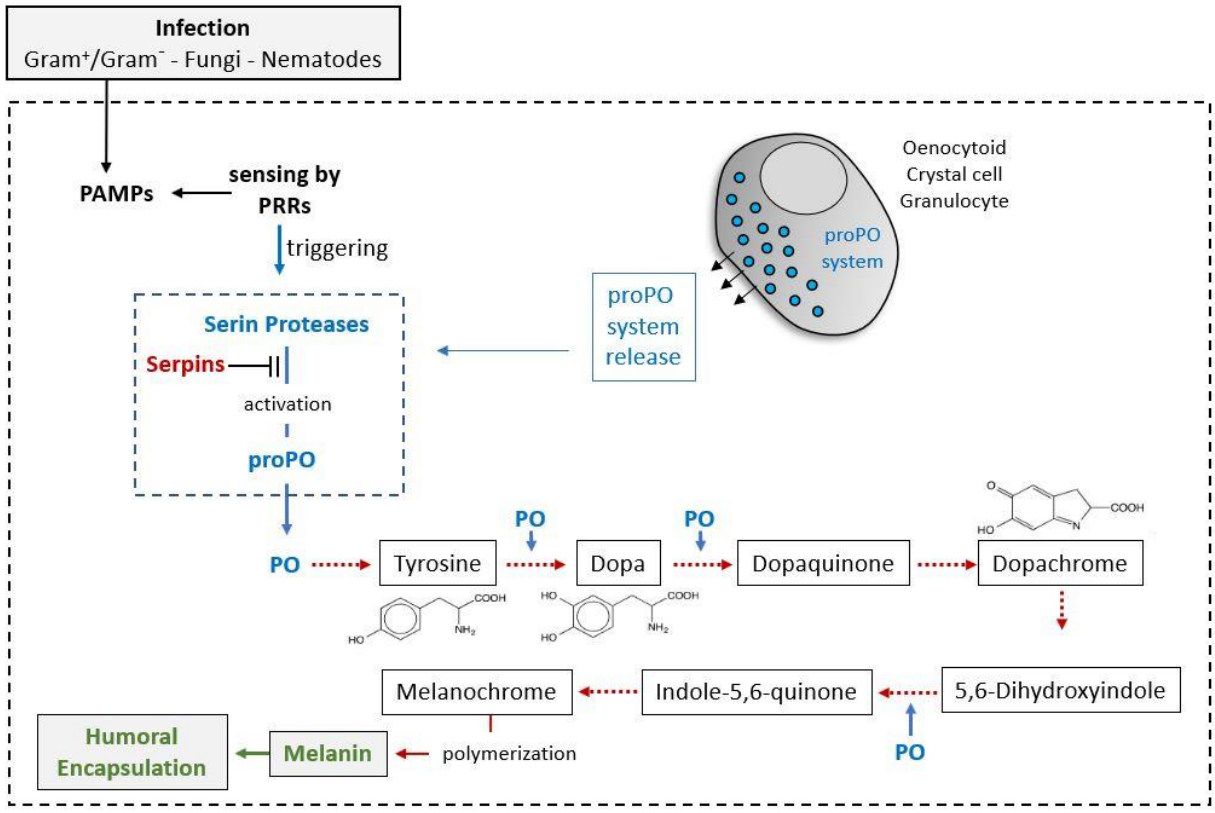


Fig. 7a - Enzymatic pathway of pro-phenoloxidase system.

oxidoreductase (PO) (Soderhall et al, 1998). Phenoloxidase is often found in the insect hemolymph in its inactive form pro-phenoloxidase (proPO), the zymogen is stored in the hemocytes and when required, secreted in the insect blood. ProPO is a polypeptide of approximately 80 kDa of molecular weight, the enzyme catalyzes the o-hydroxylation of monophenols and oxidation of phenols to quinones which then polymerize non-enzymatically to form melanin (fig. 7a) (Nappi et al, 2004).

Deposition of melanin coats is frequently observed in the encapsulation of parasites or abiotic material experimentally injected inside the hemocoelic cavity. The melanin capsule can block the absorption of nutrients by parasites and thus contribute to their killing by starvation (fig. 7b). Most reports indicate that proPO is synthetized mainly by hemocytes. The process of melanization is initiated by host soluble PRRs that bind target surfaces initiating the serine protease cascade leading to cleavage of proPO to PO and ultimately the cross-linking of proteins that produce melanin polymers. Several hemolymph PRRs are involved in the proPO system activation pathway (such as LPS-binding proteins, PGN-binding proteins, β-glucan-binding proteins etc.).

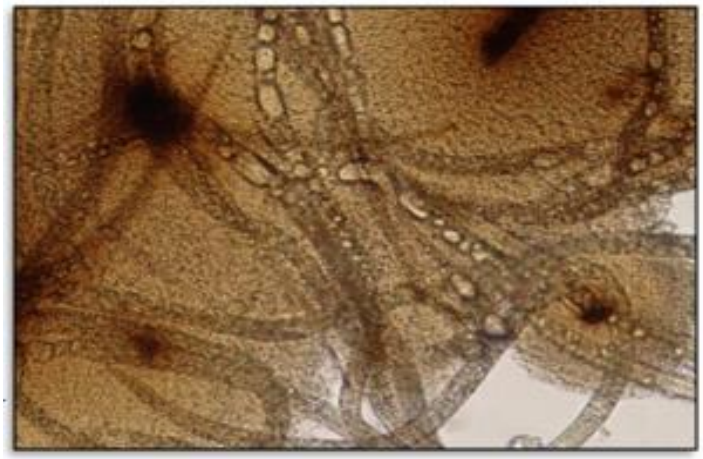


Fig. 7b - Melanin formation and free-living nematodes trapped inside melanin masses.

This enzymatic cascade system is up- or down-regulated to produce a local melanization response at a specific place and for a limited time, since its unwanted and/or massive activation could lead to a serious damage to the organism.

2.3.2 Antimicrobial Peptides (AMPs)

One of the most interesting line of defense against the pathogens invasion is the synthesis of a wide range of antimicrobial peptides (AMPs). AMPs are produced mainly in the fat bodies cells (the equivalent of the mammalian liver) during the systemic response triggered against the pathogens; they are then released into the hemolymph.

Antimicrobial peptides are gene-encoded, ribosomal synthetized polypeptides and they usually have common characteristics, most of them are all small peptides, ranging from 2 to 20 KDa. AMPs often contain the basic amino acids lysine or arginine, the hydrophobic residues alanine, leucine, phenylalanine or tryptophan and other residues such as isoleucine, tyrosine and valine.

Amphipathic Linear Peptides			
Name	**Source**	**Size (aa)**	**Activity**
Cecropin	Lepidoptera, Diptera	31–39	Gram negative/positive
Moricin	Lepidoptera	42	Gram negative/positive
Mellitin	Hymenoptera	26	Gram negative/positive
Cyclic Cysteine Rich Peptides			
Defensin	Diptera, Hemiptera, Coleoptera, Lepidoptera	32–43	Gram positive/negative
Drosomycin	Diptera	44	Fungi
Peptides Rich in Specific Amino Acids			
Drosocin	Diptera	19	Gram negative
Diptericin	Diptera	100–110	Gram negative
Attacin	Lepidoptera	214–224	Gram negative
Coleptericin	Coleoptera	74	Gram negative
Gloverin	Lepidoptera	36–261 *	Gram negative

Tab.2 - Examples of antimicrobial peptides (AMPs).

There is an increasing interest in the pharmacological application of AMPs, since antibiotic resistance to conventional antibiotics is very serious issue (Brogden, 2005). One of the most important advantage of AMPs is the global mechanism of their action which is very different from that of conventional antibiotics. When Cecropin, the first AMP, was discovered from *Hyalophora cecropia* in the 1980s by Boman, more than 200 peptides have been identified in insects.

Cecropins are widespread throughout the animal kingdom. In insects, however, cecropins have been described only in the orders of Diptera and Lepidoptera. About their chemical structures, mature cecropin peptides lack cysteine residues, are 35-39 amino acids and form two

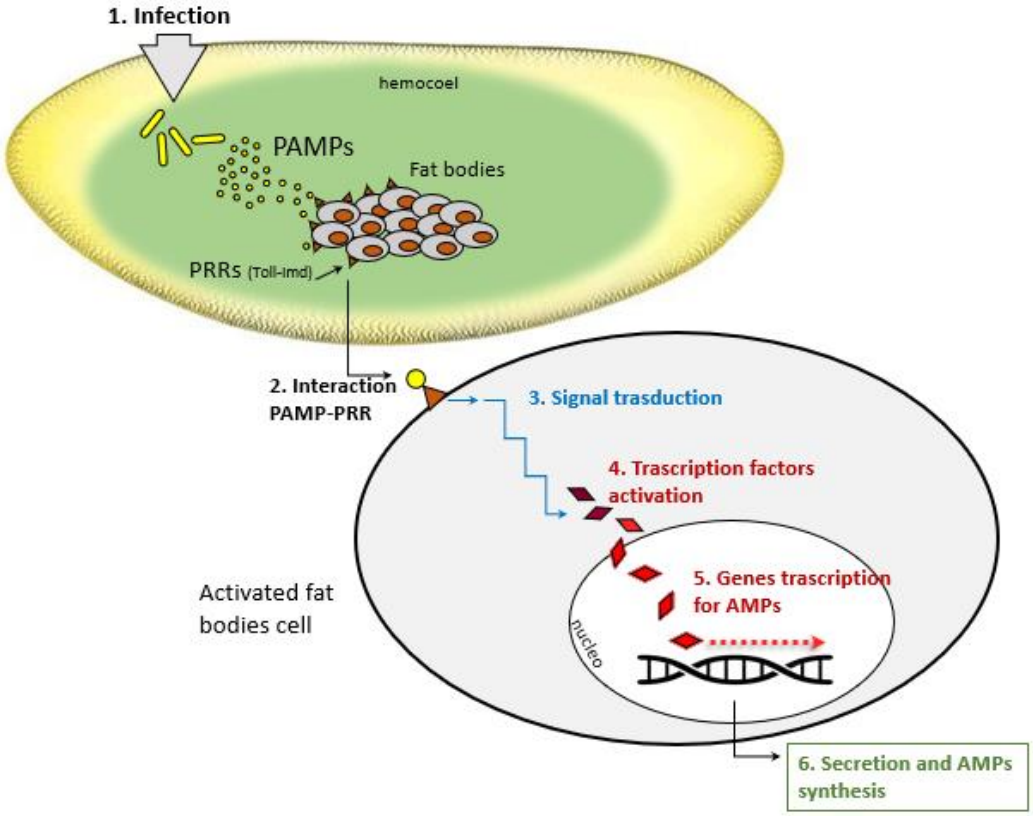


Fig. 8 - Activation scheme of Antimicrobial Peptides (AMPs) production and synthesis.

linear α-helices connected by a hinge, which integrate into the acidic cell membranes of bacteria leading to their disruption. Defensins form a unique family of cysteine-rich cationic polypeptides that are mainly effective against Gram-positive bacteria and have potent activity against some Gram-negative bacteria, fungi, yeast and protozoa (Zasloff, 2002; Bulet et al, 2004) (Tab.2).

To investigate the action and activity of AMPs, different techniques have been used. Antimicrobial peptides are able to recognize and bind to bacterial surfaces throughout the mechanism of electrostatic bonding between cationic peptides and structures on the bacterial surface (fig. 8). Once near to the microbial cell wall surface, peptides can interact with the outer bacterial membrane which contains LPS (Gram negative), and after they crossed the peptidoglycan layer, they can interact and damage the cytoplasmic membrane (Scott et al, 1999).

Different models have been proposed to explain membrane damage and permeabilization.

In the “barrel-stave model”, peptide helices from a bundle in the membrane with a central lumen, like a barrel composed of helical peptides as the staves. The hydrophobic peptide regions align with the lipid core region of the bilayer and the hydrophilic peptide regions form the interior region of the pore (fig. 9) (Yang et al, 2001).

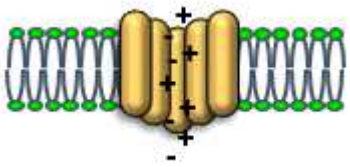


Fig. 9 - Barrel stave model: AMPs monomers assembled into the lipid bilayer create hydrophilic pores.

In the “carpet model”, peptides accumulate on the bilayers surface. This model explains the activity of antimicrobial peptides such cecropin that orientate parallel to the membrane surface. Peptides are electrostatically attracted to the anionic phospholipid head groups at numerous sites covering the surface of the membrane in a carpet-like manner. At high peptide concentrations, peptides are thought to disrupt the bilayer in a detergent-like manner, eventually leading to the formation of micelles (fig. 10) (Oren et al, 1998).

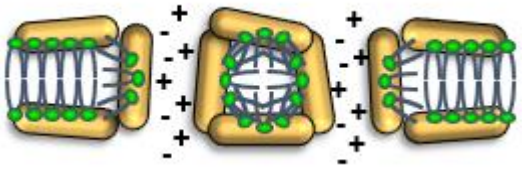


Fig.10 - Carpet model: AMPs interact with lipids destabilizing and disassembling the membrane structure.

In the “toroidal-pore model”, antimicrobial peptide helices insert into the membrane and induce the lipid monolayers to bend continuously through the pore so that both the inserted peptides and the lipid head groups line the water core. In forming a toroidal pore, the polar faces of the peptides associate with the polar head groups of the lipids. The differences between the toroidal model from the barrel-stave model are that the peptides are always associated with the lipid head groups even when they are perpendicularly inserted in the lipid bilayer (fig. 11) (Matsuzaki et al, 1996).

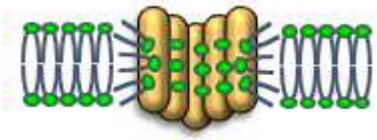


Fig. 11 - Toroidal pore model: heads of membrane lipids interact with the polar portions of the AMPs inserted into the bilayer.

2.4 Cellular defenses

Hemocytes are responsible for several defense responses in insects, among which phagocytosis, nodulation and encapsulation.

2.4.1 Phagocytosis

The phagocytosis process of foreign particles is almost the same throughout the animal kingdom, and in many of unicellular organisms, phagocytosis is predominantly used for feeding.

Phagocytosis is the process by which cells recognize, bind and ingest small particles such as bacteria and this process is probably the oldest defense mechanism against microorganism (fig. 12). This defensive process begins with the recognition by phagocytic receptors of the hemocytes that activate various signaling pathways; these signals lead to important changes in the dynamics of the plasma membrane and the cytoskeleton of specialized cells. The membrane extends pseudopods around the target, surrounding them before the engulfment. Then, membranes fuse at the distal end, assembling a new plasma membrane-derived phagosome. The phagosome fuses with lysosome to form a mature phagolysosome (fig. 12).

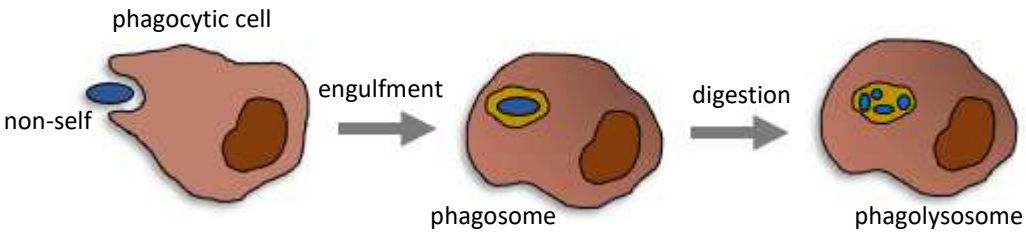


Fig. 12 - The process of phagocytosis.

This compartment has an acid environment full of hydrolytic enzyme, responsible of the digestion of the engulfed target. In insects, phagocytosis is performed by a subset of hemocytes present in the hemolymph (Strand, 2008), in Diptera and Lepidoptera professional phagocytes are plasmatocytes or granulocytes. Many studies show that phagocytosis eliminates mainly two types of targets: microorganism and particles represented by apoptotic cell fragments, this process is central during tissue remodeling and embryogenesis (Lavine and Strand, 2002; Lemaitre and Hoffmann, 2007).

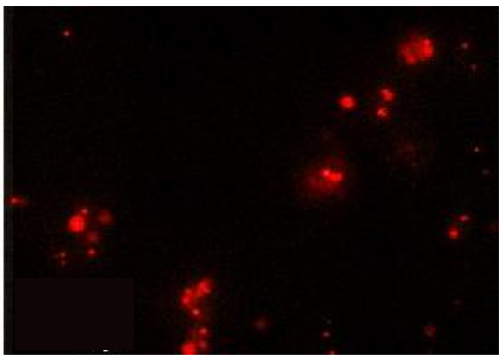


Fig. 13 - Phagocytosis evaluation by pHrodo probe

The process of phagocytosis in insect and mammals appears to be very similar; in both cases, the process is initiated after the interaction of opsonins with specific receptors at the surface of not self. Phagocytosis can also be triggered through the interaction of phagocytes membrane receptors with specific molecules, such as lipids or sugars on the microorganism cell wall (Stuart and Ezekowitz, 2005). Regarding the infection of Gram-negative bacteria, specific

lectins recognize and bind to peptidoglycans on the bacterial cell surface facilitating the binding to plasmatocytes and the phagocytosis process. At the same time the hemolymphatic enzyme, lysozyme, degrades the peptidoglycan layer releasing sugars and exposing teichoic acid and lipomannans that are recognized by lectins. This, therefore, suggests how the cellular and humoral defenses of the innate immune system cooperate in fighting infection (Lagueux et al, 2000).

2.4.2 Encapsulation

Encapsulation is a defensive response of hemocytes against large targets that cannot undergo to phagocytosis or nodulation, e.g. nematodes, parasitoids, fungi and abiotic large particles (e.g. Sephadex beads) (fig. 14) (Götz and Boman, 1985).

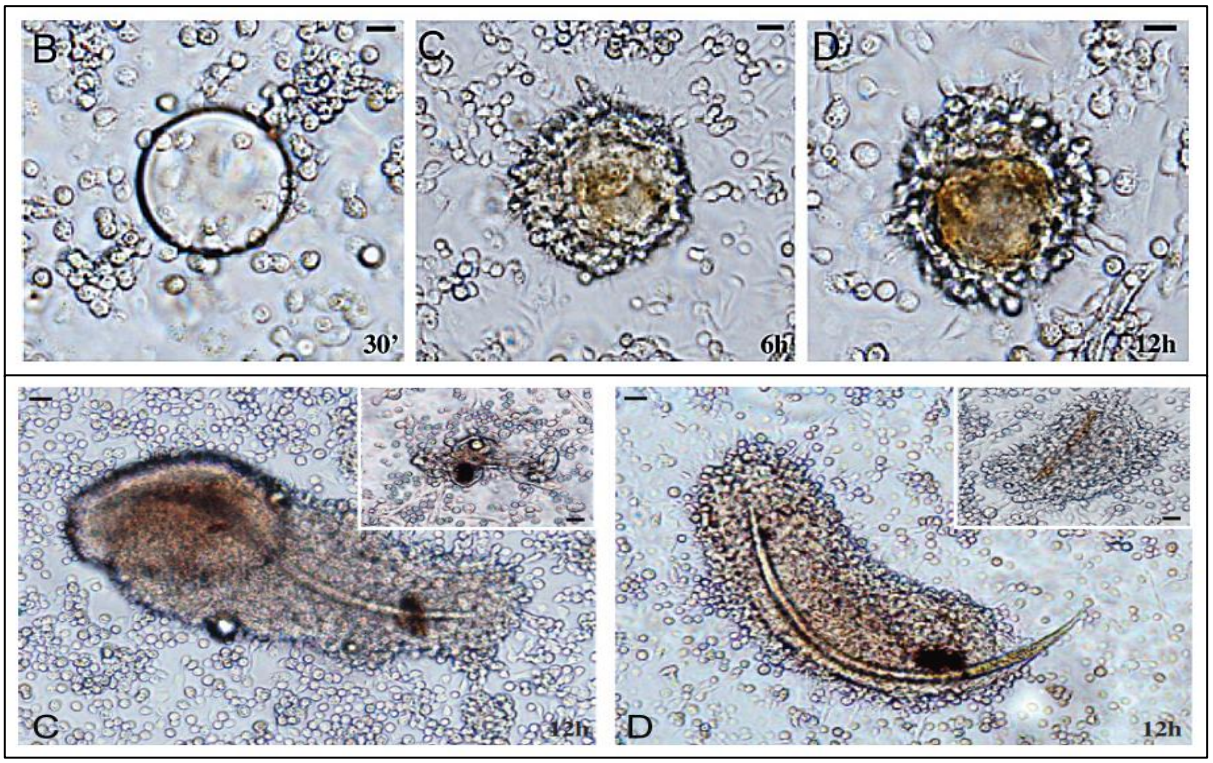


Fig. 14 - Subsequent steps in cellular encapsulation of a free-living nematode.

In insects there are generally two types of encapsulation, cellular encapsulation and melanotic humoral encapsulation. In contrast to the melanotic encapsulation process, which is always associated with phenoloxidase activity, cellular encapsulation can occur without any sign of melanization. In the cellular encapsulation, granulocytes generally interact with foreign targets then they release chemotactic components that attract plasmatocytes, the two cells populations contribute to build a multilayered capsule; within the capsule, the parasite could be killed also by the local production of cytotoxic compounds.

Based on observations in several insect species (Gagen and Ratcliffe, 1976; Peck and Strand, 1996) a common sequence of events has been proposed (fig. 15). Firstly, hemocytes contact a foreign object via random movement or directed chemotaxis, then the granular cells adhere and degranulate. Material secreted from granular cells binds to the foreign surface and to other hemocytes. Secreted molecules function also as attractant for other granular cells and plasmatocytes. Indeed, granular cells possess a signal that recruits and activates plasmatocytes to participate in the capsule formation (fig. 15).

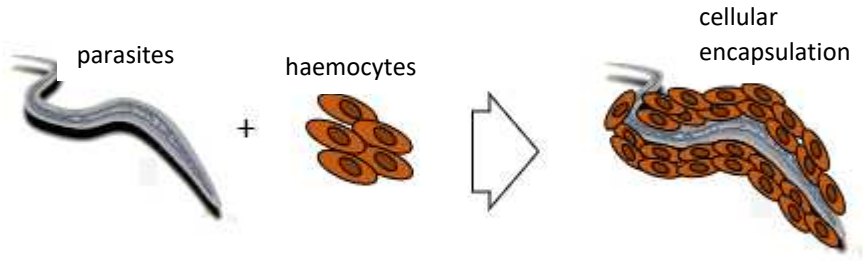


Fig. 15 - The process of encapsulation.

Granular cells attached to the target object begin to disintegrate and a second thin layer of granular cells covers the envelope that may be melanized. Furthermore, because the capsule does not grow indefinitely, the signal to recruit new hemocytes for encapsulation must decrease with each increased envelope size.

2.4.3 Nodule formation

Nodules are aggregates of hemocytes with the function to capture single cell invaders; this process (nodulation) is the main immune response to high concentrations of non-living particles, microbes, fungal spores, yeast cells or protozoa (Guzo and Stoltz, 1987; Gillespie et al, 1997). Nodules formation appears to be typical for most Lepidoptera (Ratcliffe and Gagen, 1977). For example, within a minute after injection of heat-killed bacteria in *Galleria mellonella*, the

granules within the granular cells that had randomly encountered the bacteria began to swell. These granules migrated out towards the granular cells' periphery and expelled an adhesive substance into the surrounding hemolymph, which then entrapped the bacteria. This resulted in an aggregation of granular cells and bacteria embedded in an extracellular matrix. After 5 minutes, the forming nodules were 50-100 µm in diameter. Granular cells were still undergoing degranulation as the nodule continued to enlarge. The aggregation became more compact and depositions of melanin appeared near entrapped bacteria.
The completed nodules were comprised of a central flocculent mass consisting of bacteria and the oddments of granular cells and any other hemocytes that had become entrapped, all embedded in a melanized matrix and surrounded by a multilayer of plasmatocytes. Granular cells and plasmatocytes were the only hemocytes observed to play a role in nodule formation (Ratcliffe and Gagen, 1977; Chapman, 1998).

2.5 The role of the parasite surface against the hosts

Parasites immunoevasion strategies often involve the parasite body-surface that seems to play a key role in the interaction with the host environment (Blaxter et al, 1992). Nematodes moult several times throughout their development cycle, each time they change a new cuticle molecular architecture can appear. Moreover, single species may have sharp differences in molecular organization and surface properties; this is particularly true for parasitic species (i.e. *S. feltiae*) that must interact with a potentially harmful host environment. Parasitic nematodes may easily elaborate the composition and organization of the epicuticular external layer, depending upon the environment of each species (Maizels et al, 1993).

With other surface and secreted molecules (Poliz and Philipp, 1992), the cuticle of parasitic nematodes seems to be involved in immunoevasion and suppression of host defenses, as suggested also by many authors (Akhurst and Dunphy, 1993). Thus, it is likely that nematodes body-surface play a crucial role in parasite success. The hypothesis of a role of the body-surface of parasites is based on the point that, in absence of active suppression mechanisms, the avoid of encapsulation could be achieved by means of the acquisition of a coat composed of host proteins (this process is called "molecular disguise") instead the production of modified self-antigens is conventionally defined as "molecular mimicry".

2.5.1 The nematocomplex S. feltiae – X. bovienii

In 1986 Dunphy and Webster furnished preliminary evidences of a speculative role of the epicuticle layer of *S. feltiae* in host cellular immunodepression. The paper pointed out interactions of the body-surface of the entomopathogen with *G. mellonella* hemocytes and suggested its involvement in avoiding cellular encapsulation. Authors described a partial characterization of cuticle sugars, by means of lectins specificity but, more interestingly, they assessed the role of the lipidic moiety of the epicuticle of the parasite. A simple assay based on lipase treatments of the cuticle verified that surface lipids integrity was fundamental in the escaping from hemocytes recognition; given that, they supposed that modifications of the lipidic surface resulted in changed molecular architecture of the epicuticle exposing discriminable antigens.

Considering that proPO enzymatic cascade is the faster process whenever the host is exposed to an infection of foreign, it is reasonable to assume that the parasite penetrating into insects becomes a potential target for the host prophenoloxidase activating system. Experimental evidences showed a drastic host proPO system inhibition in *G. mellonella* larvae infected with living *S. feltiae*. Otherwise, infection with free-living nematodes (*Caenorhabditis elegans*) resulted in a significant increase in proPO system activity, culminating in a marked melanization of the worm. Thus, the obligate parasite *S. feltiae* and the free-living nematode *C. elegans* (both belonging to the *Rhabditidae* order), induced opposite effects in the insect host, evading the immune system or strongly stimulate it respectively. Then, an assay exclusively with cuticle structures isolated from the worm was carried out that resulted in a drastic suppression of hemolymphatic phenoloxidase activity, moreover, these results were comparable with the experiments performed with whole parasites. The integrity of the molecular architecture of the parasite body surface seems to be essential to retain its immunodepressive properties, because chemical and enzymatic alterations of the structure often resulted in a marked loss of inhibition of the host pro-PO cascade.

Moreover, the inhibitory properties of cuticles are ascribed to the lipidic moiety of the structure. Indeed, when lipids were altered or removed by enzymatic (lipase) or chemical (methanol-chloroform) treatments, immune responses of the host were restored. Furthermore, parasite cuticular lipids (PCLs) were effective in host immune depression also when used in purified form; when injected into the host hemocoel extracted cuticular lipids induced the same

effects observed with whole cuticle (or parasite). In general, parasite survival strategies are aimed to suppress or alter host defenses; in this context, parasites often use the similar immune signaling molecules and molecular disguise mechanisms to escape host immunosurveillance, these strategies are usually referred as interference (Loker, 1994). The interference of the parasite affects both cellular and humoral components and often results in the neutralization of immune defenses (Mastore and Brivio, 2008).

2.5.2 The nematocomplex S. carpocapsae – X. nematophila

As we know, there is a mutualistic relationship between *S. carpocapsae* and his symbiont *X. nematophila*: the nematode is the vector used by the bacteria to penetrate and infect the larvae. Killing the host insect, the nematode/bacteria complex converts the larva body into an abundant source of food for the second generation of nematodes at the early stages of development; moreover, by preventing further microbial infections on the corpse it excludes possible trophic competitors (Thaler et al, 1997; Mitani et al, 2004).

The localization of the bacteria in the nematode affects some areas of the worm gut called vesicles, *X. nematophila* settles in vesicles of *S. carpocapsae* (Akhurst, 1983) using for adhesion and migration the structures and proteins placed on the outer surface of the bacterial wall (Martens et al, 2005). These surface structures, in addition to being involved in this process of colonization, interact with components of the host immune system. The bacterium also seems to play an important role in the development of the nematode's sexual organs (Poinar, 1966).

The nematode penetrates the insect through physical openings, including mouth, anus, respiratory stigmas and possible lacerations of the cuticle. Once inside the host has three possible strategies to counteract the immune system of the insect: evade the immune system by colonizing organs with low immunocompetence, or camouflaging at the molecular level, to avoid recognition by PRRs, finally the complex can interfere with the immune component of the host, directly inhibiting some essential processes of the immune response (Brivio et al, 2005).

After penetration, the nematode releases symbionts in the hemolymph (1-2 hours later) which replicate rapidly and secrete toxins and toxic factors (Goodrich-Blair et al, 2007; Waterfield et al, 2009; Rodou et al, 2010). Many studies have suggested that the presence of *Xenorhabdus* may induce host immune response (Peters and Ehelers, 1997), although there is evidence that symbiont secretions have suppressive effects on cellular immune response (Dunphy and Webster, 1988).

X. nematophila has two vital phases, defined as I and II. In phase I (virulent) the bacterium is highly infectious and has numerous structures on the surface used to interact with cells and tissues of the host insect and responsible for motility. In phase II the bacterium is not virulent and appears to have no external structures. On the bacterial wall of *X. nematophila* in phase I there are

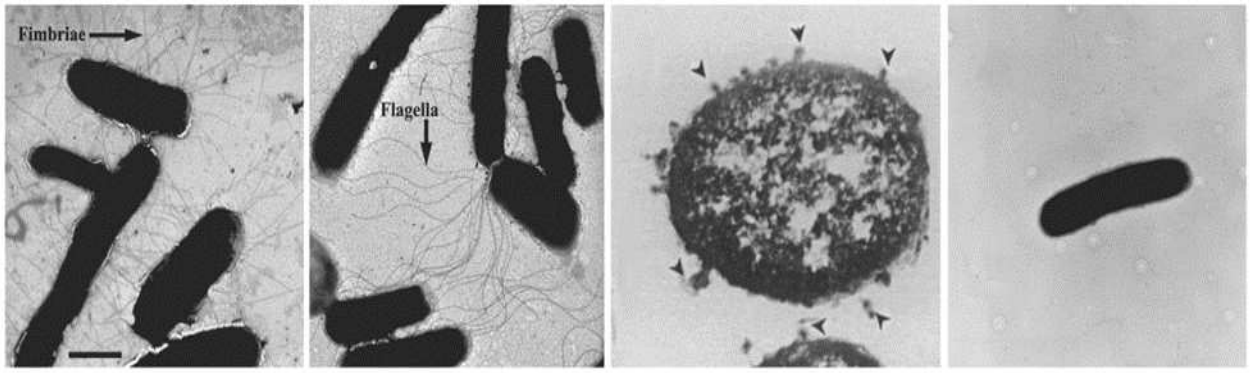


Fig. 16 - External structures of Xenorhabdus nematophila.

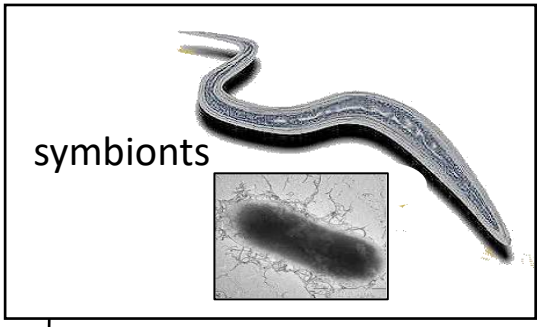
different structures, such as piles/fimbries and external vesicles (fig. 16). Fimbrias are protein structures that allow the bacterium to adhere to the substrate and once in the hemolymph they appear to be responsible for the non-recognition of *X. nematophila*.

Some of the components of hair/fimbria appear to be responsible for agglutination on both mammalian erythrocytes and insect hemocytes (Moreaux et al, 1995). Pore formation phenomena in the hemocyte membranes have also been described (Khandelwal et al, 2004; Banerjee et al, 2006). It was also observed that the described structures are present on *X. nematophila* only when the bacterium in phase I, is in mutualistic relation with the nematode or is released into the host hemocoel (Binnington et al, 1993).

Given that, our goal was to study of the immunodepressive properties the protein pool called XoP (*Xenorhabdus* Outer Proteins) extracted from the bacterial wall of *X. nematophila*, symbiont of the nematode *S. carpocapsae*. The effects of XoP were evaluated against a model insect (*Galleria mellonella*) and the effects on the activity of the proPO system and on the phagocytic activity of the host blood cells were examined, and finally their cytotoxic activity on the hemocytes population of the insect was tested.

Project topics

Bioinsecticides
(nematodes and bacteria)



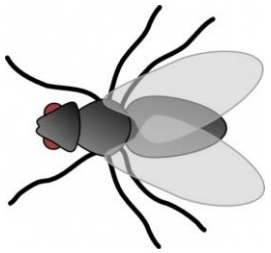
Infection

Elusive strategies

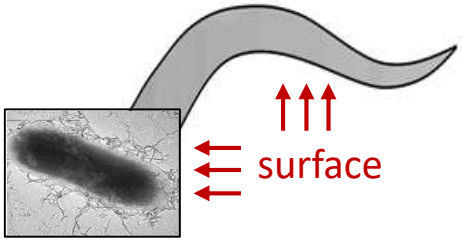
Counteraction

Immune response

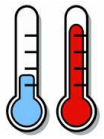
Insect Hosts



Role of parasites and symbionts surface



Influence of environmental temperature



CHAPTER 3

Paper summary:

Surface protein components from entomopathogenic nematodes and their symbiotic bacteria: effects on immune responses of the greater wax moth, Galleria mellonella (Lepidoptera: Pyralidae).

3.1 Aims of the project

Our goal was to study the immuno-depressive properties of the protein pool arbitrarily named SoP (*Steinernema* Outer Protein) and *XoP* (*Xenorhabdus* Outer Proteins) extracted respectively from the surface of the nematode *S. carpocapsae* and from the wall of *X. nematophila*, its bacteria symbiont. The effects of both extracted protein pools (SoP and XoP) were evaluated against an insect (*Galleria mellonella*); particularly, we have assessed if these compounds affected the proPO system activity and the phagocytic capability of the host hemocytes. Finally, the potential cytotoxic activity on the host hemocytes was tested.

3.1.1 Introduction

When the EPNs penetrate in the haemocoel, they immediately encounter the host immune system; thus, their surface molecules interact with both humoral and cellular receptors present in the host hemolymph. For this reason, we have planned to isolate the protein fractions from the epicuticle/cuticle of nematodes (SoP) and from the bacterial cell wall (XoP) (Fig. 17), using high-salt extraction protocols. The literature clearly reports that *Xenorhabdus* spp., when in the virulent phase I, possess various surface structures (pili/fimbriae and flagella) that interact with the host body; these surface structures affect

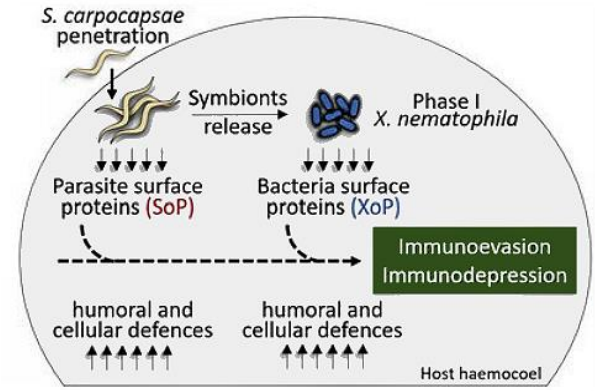


Fig. 17 - Scheme of the effects of SoP and Xop on the immune response of an insect host

the recognition by hemocytes enabling the bacterium to elude both phagocytosis and nodulation processes. Moreover, the OMVs (outer membrane vesicles) present on the bacterial surface, contain virulence factors which are released and contribute to the lethal effects of *X. nematophila.* Then, we examined the cytotoxic effects of SoP and XoP on host hemocytes and a potential interference with phagocytosis mediated by immunocompetent cells.

3.1.2 Materials and methods

As insect model, we used *G. mellonella* larvae reared on a sterile food mixture. As EPNs we used the nematode *S. carpocapsae* and its symbiont *X. nematophila*. Samples of both live and dead bacteria were prepared for assays. To obtain host hemocytes, the hemolymph samples were flushed out in Eppendorf tubes and, after centrifugation, hemocytes were washed twice with Grace's insect medium. CFF (cell-free fraction) was obtained from whole hemolymph by centrifugation and the supernatant was recovered and clarified by several centrifugations at high gravity.

To verify the presence of surface structures, bacteria were prepared for the electron microscopy. Components of the surface of both live or dead nematode (SoP) and live or dead bacteria (XoP) were isolated through a high ionic strength buffer to elute surface proteins soluble. About the measurements of hemocyte viability of the insect model, were carried out in vitro assays using the MTT colorimetric assay. In addition, to investigate the effects of the whole nematode and the isolated XoP on the host phagocytosis process, we performed an in vivo double-infection experiment (for details see the paper below). Finally, the proPO system activity in the CFF of *G. mellonella* larvae was examined in vitro and monitored by spectrophotometric analysis.

3.1.3 Summary of results

Live parasites and their secretions showed a cytotoxic effect. In particular, the cytotoxic effect of XoP when extracted from live *X. nematophila* was evident; this effect was instead reduced when the insect cells were treated with XoP derived from dead bacteria.

In the evaluation of hemocyte phagocytosis, the ability of host hemocytes to phagocytose was assessed after the injection of the nematocomplex *S. carpocapsae* and we saw a significant difference between controls and treatments tested. The presence of nematodes in a short time

following infection did not significantly modify the phagocytosis level. However, a significant effect was observed when parasite infection was protracted for a long time (3h).

In the analysis of the proPO activity in the hemolymph, the time-course shows that in particular the XoP did not inhibit the host phenoloxidase enzyme, and thus they seem to strongly trigger the host proPO system activity. Instead, a marked inhibition was observed when hemolymph was incubated together with live bacteria (for details see the paper below).

The figure below summarizes the effects induced by the parasites, its symbiont bacteria and their secretions or surface structures on the immune system of the target insect (fig. 18).

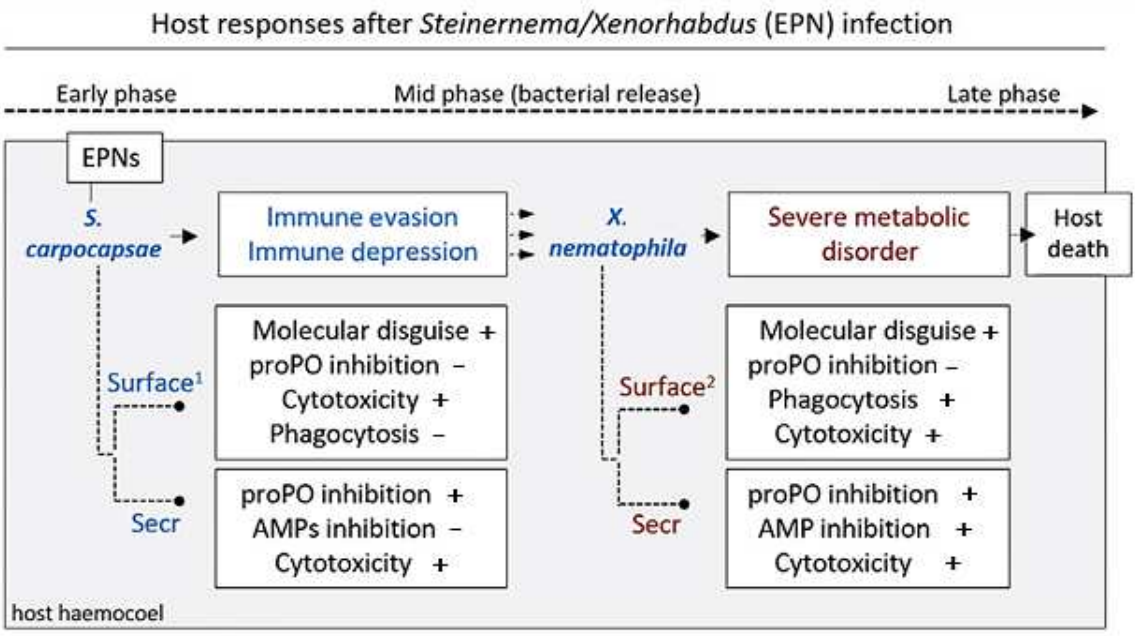


Fig. 18 - A summary of the main effects induced by parasites, symbionts, surface compounds and secretions. Surface[1]: SoP; Surface[2]: XoP; +: interference with the process; -: no effect on the process.

Research Article

Received: 2 November 2017 Revised: 6 February 2018 Accepted article published: 8 March 2018 Published online in Wiley Online Library: 14 April 2018

(wileyonlinelibrary.com) DOI 10.1002/ps.4905

Surface protein components from entomopathogenic nematodes and their symbiotic bacteria: effects on immune responses of the greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae)

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Abstract

BACKGROUND: *Steinernema carpocapsae* is a nematocomplex widely used as an alternative to chemicals for the biological control of insect pests; this nematode is symbiotically associated with the bacterium *Xenorhabdus nematophila* and both contribute to host death. The architecture and functions of structures and molecular components of the surface of nematodes and their symbiont bacteria are integral to early interactions with their hosts; thus, we assessed the role of protein pools isolated from the surface of *S. carpocapsae* and from phase I *X. nematophila* against *Galleria mellonella*.

RESULTS: Using high-salt treatments, we isolated the surface proteins and assayed them on *G. mellonella* haemocytes; haemocyte viability and phagocytic activity were investigated in the presence of surface proteins from nematodes or bacteria. Proteins from live *S. carpocapsae* possessed mild cytotoxicity on the haemocytes, whereas those from live *X. nematophila* markedly affected the host cells' viability. Bacterial proteins inhibited phagocytic activity, although they strongly triggered the host proPO (prophenoloxidase-phenoloxidase) system.

CONCLUSION: Nematocomplex surface compounds play a key role in immunoevasion/depression of insect hosts, causing a severe physiological disorder. Natural compounds newly identified as active against pests could improve the pest management of species potentially harmful to plants in urban green spaces and agriculture.

Keywords: pest control; *Steinernema carpocapsae*; *Xenorhabdus nematophila*; *G. mellonella*; insect immunity; entomopathogens

1 INTRODUCTION

Entomopathogenic nematodes (EPNs) are symbiotic complexes commonly used as bio-insecticides in biological control of insect pests. As a result of cooperation between the parasite and its symbiotic bacteria, they have lethal effects on their insect targets.[1] Well-known examples of such symbiotic complexes are nematodes of the genus *Steinernema* that associate with bacteria of the genus *Xenorhabdus*.

The success of the parasitisation results mainly from immunological disabling induced by both the nematode and its symbionts when released inside the host haemocoel.[2–4]

Many works have addressed the roles of the two factors separately, focusing on the effects of toxic compounds secreted by the parasite[5–8] or released by the bacteria, such as toxins, proteases, lipases and antibiotics.[9,10] Many actively secreted molecules have been assayed for their effects on the immune system and for their lethal properties,[11,12] suggesting that the host damage is caused by multifaceted action carried out individually or synergistically by these molecules.

Regarding the relationships between EPN complexes and their hosts, we cannot disregard the role played by the body surface of the invaders, as the first contact with the host immune system always involves supramolecular and/or molecular factors present

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on the surface of the invaders and free or cell-associated receptors of the host.[13,14]

Many reports have focused on the role of the body surface of parasites in the relationship with their hosts,[15–18] and some authors suggested a key role for compounds derived from the EPN's cuticle, epicuticle or additional outer coats,[14,19–22] suggesting that the body surface of nematodes is effectively involved in processes of evasion and suppression of host immune defences.

When the symbionts of EPNs are released in the host haemocoel, they immediately come into contact with the host immune system. Therefore, before any secretion processes, their surface molecules interact with both humoral and cellular receptors present in the host haemolymph.

This mechanism has already been confirmed by many studies that have shown that *Xenorhabdus* spp., when in phase I, known as the virulent phase, possess various surface structures that interact with the host body.[23] Among these surface structures, pili/fimbriae and flagella, or their molecular constituents, affect the recognition of haemocytes, preventing phagocytosis and nodulation processes.[24] Flagella, and their main constituent flagellin, are responsible for adhesion to and invasion of host tissues as well as the avoidance of phagocytosis, and thus all these structures play a role in interference with host defence processes.[10,25] Moreover, outer membrane vesicles (OMVs) present on the bacterial surface contain virulence factors, such as proteases, lytic factors and phospholipase C, which are released normally or under stress conditions and contribute to the larvicidal activity of *Xenorhabdus nematophila* (Enterobacteriaceae).[26]

In the presence of an infection, insects, in most cases, trigger innate immune responses implemented by both immunocompetent cells and molecular factors that recognise and neutralise almost any foreign invader.[27,28] The insect immune system is arbitrarily divided into humoral and cellular defences: humoral defences include antimicrobial peptides,[29] oxygen- or nitrogen-reactive intermediates, and the enzymatic cascades that regulate coagulation or melanisation (the proPO system) in the haemolymph.[30,31] Cellular defences, comprising haemocyte-mediated immune responses, involve the action of circulating cells, namely granulocytes and plasmatocytes. These cells are involved in cellular defensive mechanisms, such as phagocytosis, nodule formation, encapsulation, melanisation and synthesis of antimicrobial peptides.[32,33]

However, the success of EPN parasitisation is strictly related to the ability of the parasite and its symbionts to neutralise the recognition and consequent effector processes triggered by the presence of foreign bodies inside the haemocoel.

In many studies, the ability to overcome the host immune system has been primarily attributed to the action of compounds secreted by EPNs. In contrast, the aim of this study was to investigate the effects induced by proteins from the surface of both *Steinernema carpocapsae* (Nematoda: Rhabditidae) and its symbiont *X. nematophila* on *Galleria mellonella* (Lepidoptera: Pyralidae) larvae.

We isolated protein fractions from the epicuticle/cuticle of nematodes [*Steinernema* outer proteins (SoP)] or from the bacterial cell wall [*Xenorhabdus* outer proteins (XoP)] using high-salt treatments and analysed them by electrophoresis. We then examined their cytotoxic effects on host haemocytes and their interference with phagocytosis by immunocompetent cells. Finally, we analysed the role of *X. nematophila* and of its surface proteins in the modulation of the host proPO system.

2 MATERIALS AND METHODS

2.1 Insect host and entomopathogens

As an insect host, we used *G. mellonella* larvae reared on a sterile food mixture; only healthy late-stage caterpillars were selected for the experiments.

As entomopathogens we used the nematode *S. carpocapsae* (UK strain) and its symbiont *X. nematophila*; parasites were purified from the commercially available product Entonem® provided by Koppert Biological Systems (Koppert B.V., AD Berkel en Roderijs, the Netherlands). Briefly, nematodes were separated from the inert carrier material by rinsing several times with tap water, then centrifuged at 200 ***g***, for 10 min, on a discontinuous (25%–50%–75% w/v) sucrose gradient using an Eppendorf 5804R (Eppendorf, Hamburg, Germany). Clarified parasites were collected at the interface between 25 and 50% sucrose in the gradient and washed with phosphate-buffered saline (PBS) sterilised buffer (138 mM NaCl, 2.7 mM KCl and 10 mM Na_2HPO_4/KH_2PO_4, pH 7.4) from Serva (Heidelberg, Germany) and centrifuged (700 ***g*** for 10 min) several times. Infective juvenile-stage (IJ) nematodes were surface-sterilised with 0.5% sodium hypochlorite for 10 min on a rotary shaker (Celbio SpA, Milan, Italy) at room temperature, then washed several times with PBS buffer. Aliquots of *S. carpocapsae* were cold-killed in glycerol/PBS (1:1 v/v) for 48 h at −20 °C and the mortality was verified under a SZX10 stereomicroscope (Olympus Optical Co., Tokyo, Japan); before assays, dead nematodes were washed extensively with PBS buffer.

To obtain the symbiont bacterium *X. nematophila,* larvae of *G. mellonella* were infected with IJ *S. carpocapsae*, and at 24 h post infection dead larvae were surface-sterilised in 70% ethanol for 10 min and air-dried for 5 min, and then they were punctured with needles, and drops of haemolymph were streaked with inoculation loops onto nutrient agar plates containing NBTA (40 g L^{-1} nutrient tryptone soya agar, 25 mg L^{-1} bromothymol blue powder and 40 mg L^{-1} 2,3,5-triphenyl-tetrazolium chloride, from Sigma Chemicals, St Louis, MO, USA). Plates were incubated at 28 °C in the dark for 24 h, and then a single colony of bacteria was selected and streaked onto a new plate of NBTA. Subculturing was continued until colonies of uniform size and morphology were obtained. The pathogenicity of isolated bacteria was confirmed by injection into *G. mellonella* larvae.

The bacterial concentration was estimated by spectrophotometric reading absorbance ($\lambda = 600$ nm) using a Jasco V-560 (Jasco, Easton, MD, USA), and the phase of bacteria was assessed by analysing motility, using an Olympus IX51 microscope (Olympus Optical Co., Tokyo, Japan), swarming capability and colony blue staining on NBTA plates. Samples of both live and dead *X. nematophila* were prepared for assays; dead bacteria were obtained by heat treatment at 60 °C for 2 h in a thermostatic bath, and the absence of bacterial growth was verified by overnight culture. Bacterial cultures were washed in phosphate buffer for the haemocyte cell viability and phagocytosis assays. All materials and buffers were sterilised using an autoclave or 0.22-μm filters; all assays were performed at 4 °C under sterile conditions when required.

2.2 Collection of host haemolymph, plasma and haemocyte cultures

In all experiments, insect larvae were previously surface-sterilised with 70% ethanol, before bleeding. To avoid undesired activation of prophenoloxidase, Mead anticoagulant buffer (98 mM NaOH, 145 mM NaCl, 17 mM EDTA and 41 mM citric acid, pH 4.5; Sigma

Chemicals) was added to the haemolymph and all procedures were carried out at 4 °C.

To obtain host haemocytes, haemolymph samples were flushed out in Eppendorf tubes and diluted 1:1 (v/v) with anticoagulant buffer; then, cells were pelleted by centrifugation at 300 ***g*** for 10 min at 4 °C, haemocytes were washed twice with Grace's insect medium (Sigma Chemicals) plus 10% fetal bovine serum (Sigma Chemicals), 1% glutamine and 1% antibiotic-antimitotic solution (Sigma Chemicals), and finally cells were cultured at a concentration of about 2 x 10^4 cells/microwell (in 96-well flat-bottom culture plates; Corning Inc., NY, USA).

Cell-free plasma was obtained from whole haemolymph by centrifugation at 200 ***g*** for 10 min at 4 °C, and the supernatant was recovered and clarified by several centrifugations performed at 3000 ***g*** for 10 min at 4 °C and finally filtered using 0.22-μm filters.

2.3 Isolation of *X. nematophila* outer proteins (XoP) and bacterial secretions

Components of the surface of the cell wall of live or dead *X. nematophila* (XoP) were isolated from 3 L of bacterial cultures; briefly, *X. nematophila* overnight cultures were centrifuged at 1600 ***g*** for 15 min at room temperature, and collected pellets were washed several times with phosphate buffer (61 mM K_2HPO_4 and 38 mM KH_2PO_4, pH 7; Sigma Chemicals) and centrifuged at 1600 ***g*** for 15 min at room temperature to remove residual medium. Pellets from both live and dead bacteria were treated for 20 min, at room temperature, with high ionic strength buffer (500 mM NaCl in 10 mM Tris–HCl, pH 7.2) to elute surface proteins soluble in high salts. Samples were centrifuged at 1200 ***g*** for 20 min, at room temperature, and supernatants were recovered and filtered using the 0.22-μm Corning filter system (Corning Inc.). To obtain XoP pools, filtered samples were centrifuged using a Beckman L-80 Ultracentrifuge with 50.2 TL rotor (Beckman Coulter, Brea, CA, USA), at 37000 ***g*** for 40 min at 10 °C, supernatants were removed and finally XoP pellets were dried under gentle nitrogen flow and stored at −80 °C. Total proteins were estimated by the Bradford protein assay calibrated on bovine serum albumin (BSA).

For cell viability and phagocytosis activity, all samples were resuspended in sterile PBS (138 mM NaCl, 2.7 mM KCl and 10 mM Na_2HPO_4/KH_2PO_4, pH 7.4), and for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, samples were denatured in Laemmli buffer.[49] Bacteria treated with high ionic strength buffer (stripped bacteria) were washed several times with PBS buffer before assays.

Secretions of *X. nematophila* were obtained from overnight cultures; 10^3 CFU/mL of bacteria were extensively washed in phosphate buffer, and then incubated in Grace's insect medium for 24 h at room temperature under shaking, and finally bacteria were removed by pelleting and supernatants were filtered (0.22 μm). Aliquots of secretions were immediately used or stored at −80 °C.

2.4 Phenotypic characterisation of *X. nematophila*

Bacterial phenotypisation was performed on plates based on NBTA growth, swarming and swimming activities. *Xenorhabdus nematophila* cultures were grown overnight at 30 °C in dark conditions under shaking, 10 μL of the suspension was streaked onto NBTA plates (1% or 0.8% agar plates), and bacteria were incubated overnight at 30 °C. Phenotypic characterisation was also carried out with stripped *X. nematophila*.

2.5 Transmission electron microscopy (TEM) observations of *X. nematophila*

To verify the presence of surface structures of untreated and stripped *X. nematophila*, bacteria were processed by phosphotungstic acid negative staining, and then analysed using a Philips/FEI (Morgagni) electron microscope (FEI, Hillsboro, OR, USA). One colony of swarming *X. nematophila* was scraped from soft agar plates, resuspended in 300 μL of PBS and centrifuged at 2000 *g* for 10 min at 20 °C; bacterial pellets were washed several times in PBS buffer to resuspend cells and remove agar particles. Aliquots of bacteria were incubated with high ionic strength buffer for 20 min under shaking, and samples were centrifuged, washed to remove the saline buffer and resuspended in 50 μL of PBS. For negative staining, grids were floated on the top of a drop of 15 μL of bacterial suspension, 15 μL of water and 15 μL of 0.8% phosphotungstic acid for 60 s and then transferred to a water drop (60 s), and finally to a 0.8% phosphotungstic acid drop (60 s). Excess liquid was removed from the grids using adsorbent paper. Grids were examined by electron microscopy.

2.6 Isolation of *S. carpocapsae* outer proteins (SoP) and its secretions

To remove cuticular outer compounds from *S. carpocapsae*, 2 g of purified nematodes (live and cold-killed) was incubated with high-salt buffer for 1 h at room temperature under shaking. Nematodes were centrifuged at 700 ***g*** for 5 min at 20 °C, and supernatants were recovered and filtered (0.22 μm). Samples were dialysed overnight at 4 °C against 10 mM Tris–HCl, pH 7.2, and for a further 12 h against ultrapure water, and finally samples were lyophilised. Total proteins were estimated using the Bradford protein assay. All samples were resuspended in sterile PBS for cell viability assays or in Laemmli buffer for SDS-PAGE analysis.

To obtain parasite secretions, a pellet (2 mL) of sterilised *S. carpocapsae* was incubated in 2.5 mL of Grace's insect medium supplemented with 10% cell-free plasma of *G. mellonella* for 1 h at room temperature under shaking. Samples were centrifuged at 700 ***g*** for 10 min at 20 °C and supernatants were recovered and filtered (0.22 μm); aliquots of secretions were immediately used or stored at −80 °C.

2.7 Electrophoretic analysis (SDS-PAGE)

To analyse the protein pattern of samples from the surface of dead or live *X. nematophila* (XoP) and *S. carpocapsae* (SoP), we performed one-dimensional electrophoresis separations. SDS-PAGE (10%) gels were run using a slight modification of Laemmli's method.[34] Both XoP and SoP extracts were extensively dialysed against MilliQ ultrapure water (Burlington, MA, USA), lyophilised and resuspended in Laemmli's buffer. Aliquots, 50 μg/well of surface proteins from 10^5 nematodes and 50 μg/well of surface proteins from bacterial cultures (obtained as described above), were loaded onto a slab gel in a Bio-Rad Protean IIxi cell (Bio-Rad Labs, Hercules, CA, USA) and separated overnight at 30 V (constant voltage). Protein patterns were revealed by silver staining and molecular weight determination was carried out by the method of Weber and Osborn.[35]

2.8 Cell viability assays

Measurements of haemocyte viability of *G. mellonella* were carried out *in vitro* using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, in the presence of various compounds and pathogens. Aliquots of XoP from live and dead

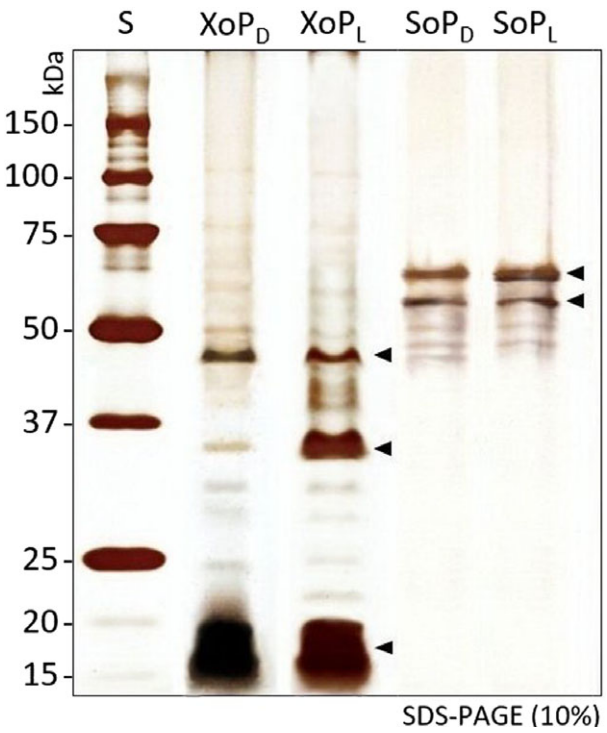

Figure 1. Analytical electrophoresis (SDS-PAGE) of samples eluted from the surface of *X. nematophila* (XoP) and *S. carpocapsae* (SoP). XoP patterns from dead (XoP$_D$) or live (XoP$_L$) bacteria show three main bands ranging from molecular mass 17 to 46 kDa and some minor components; the XoP patterns are basically comparable. The SoP patterns (SoP$_D$ and SoP$_L$, from cold-killed and live nematodes, respectively) show two main bands (54 and 69 kDa) and some minor components. For each sample, 50 μg of total protein solution was loaded onto the gels. Standard molecular weights were broad range protein markers from Bio-Rad. XoP$_D$, outer proteins from dead *X. nematophila* bacteria; XoP$_L$, outer proteins from live *X. nematophila* bacteria; SoP$_D$, outer proteins from dead *S. carpocapsae* nematodes; SoP$_L$, outer proteins from live *S. carpocapsae* nematodes.

bacteria (150 ng/μl, final concentration), live (10^3 CFU/well), dead (10^5 CFU/well), and stripped *X. nematophila* (10^3CFU/well) and *X. nematophila* secretions (150 ng/μl, final concentration) were added to haemocyte cultures to assess cytotoxic effects. A cell suspension was added to each well (96-well microwell plates) at a density of 2×10^4 cells/well, and after 1 h the medium was removed and replaced with 200 μL of fresh Grace's medium. Aliquots of the samples described above were added to wells, and all treatments were performed for 24 h; after incubation, 100 μL of MTT solution (2 mg/mL) was added to each well and plates were incubated for 2.5 h at 26 °C in the dark. Then, medium was removed and 120 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals, and plates were incubated for 15 min at 37 °C under shaking in dark conditions. The amount of formazan was measured by recording the absorbance at 570 nm in a iMark Bio-Rad (Bio-Rad Labs) plate-reader spectrophotometer. PBS buffer or dithiothreitol was added to cells as a negative or positive control. MTT assays were carried out also in the presence of aliquots of SoP (150 ng/μl, final concentration), live and dead nematodes (5–10/well) and *S. carpocapsae* secretions (Sc_{sec}; 150 ng/μl). For each assay, five replicates per condition were carried out. Moreover, all assays were replicated five times. Cell viability was calculated as the mean of treatment absorbance × (mean of control absorbance)$^{-1}$ × 100. In all assays, we assumed 100% cell viability as a control value.

2.9 Phagocytosis assays

To investigate the effects of isolated XoP on the host phagocytosis process, we performed *in vivo* double-injection experiments. Briefly, 10 μL of XoP (1 μg/μl) was injected into *G. mellonella* larvae using a Hamilton gas-tight syringe (Hamilton, Reno, NE, USA) with a 0.21-mm needle. After 1 h, larvae were re-injected with 10 μL of pHrodo™ Red *Staphylococcus aureus* BioParticles® Conjugate (2 mg/mL) (Molecular Probes, Eugene, OR, USA) and after 2 h, *G. mellonella* larvae were bled and haemocytes were isolated. Haemocytes were washed twice with PBS buffer (pH 7.4), resuspended in 1 mL of Grace's medium and cultured at 26 °C in dark conditions. After 45 min of culture, phagocytosis activity was evaluated using an Olympus IX51 fluorescence microscope equipped with a DXM1200F Nikon digital camera (Nikon Instech Co., Ltd, Tokyo, Japan). Five photo-frames for each microscope slide were counted to determine the average. Phagocytosis activity was calculated as the number of phagocytosing cells × (number of total cells)$^{-1}$ × 100. As controls, host larvae were pre-injected with 10 μL of stripped *X. nematophila* (10^3 CFU) or 10 μL of PBS buffer, and after 2 h larvae were re-injected with pHrodo-*S. aureus*.

Phagocytosis assays were also carried out in the presence of live *S. carpocapsae; G. mellonella* larvae were pre-injected with 10–15 live nematodes and at 1 or 3 h after the first injection, larvae were re-injected with pHrodo-*S. aureus*. As a control, host larvae were pre-injected with 10 μL of PBS buffer. After 2 h, haemocytes were isolated and cultured for 45 min at 26 °C in dark conditions, and finally phagocytosis activity was assessed as described above. All assays were replicated five times.

2.10 proPO system activity in the presence of XoP from *X. nematophila*

proPO system activity in cell-free haemolymph of *G. mellonella* larvae was monitored by spectrophotometric analysis; the activity was examined *in vitro* in the presence of both isolated XoP (from live bacteria) and *X. nematophila*. Cell-free fractions were obtained by two low-speed centrifugations (200 ***g*** for 10 min at 4 °C) of whole haemolymph from healthy larvae. Time courses were recorded with 5 μL of haemolymph plus 5 μg of XoP, or live bacteria (1×10^3), added to 1 mL of 8 mM L-Dopa in 10 mM Tris–HCl, pH 7.2. Absorbance changes (ΔA 490 nm 5 min^{-1}, at 20 °C) attributable to dopachrome formation were recorded using a Jasco V560 double-beam spectrophotometer, as a control, and the basal activity of the proPO system was evaluated in haemolymph samples without activators or inhibitors.

2.11 Data analysis

For cell viability assays, absorbance values from five replicates per condition and from five independent experiments were processed using a two-way analysis of variance (ANOVA). The ANOVA was followed by a Tukey honest significant difference (HSD) post hoc test to evaluate significant differences between treated samples and related controls as well as among treatments.

Data obtained from phagocytosis assays were transformed by arcsine square-root transformation, and then were analysed using a two-way ANOVA followed by a Tukey HSD test. Also, in this case values are the average (+ standard deviation) from five replicates per condition and from five independent experiments. Differences were considered significant when $P < 0.05$.

For proPO system assays, absorbance average values and standard deviations were calculated from three replicates at each time. All the statistical analyses were performed using XLSTAT2011 software (Addinsoft, New York, NY, USA).

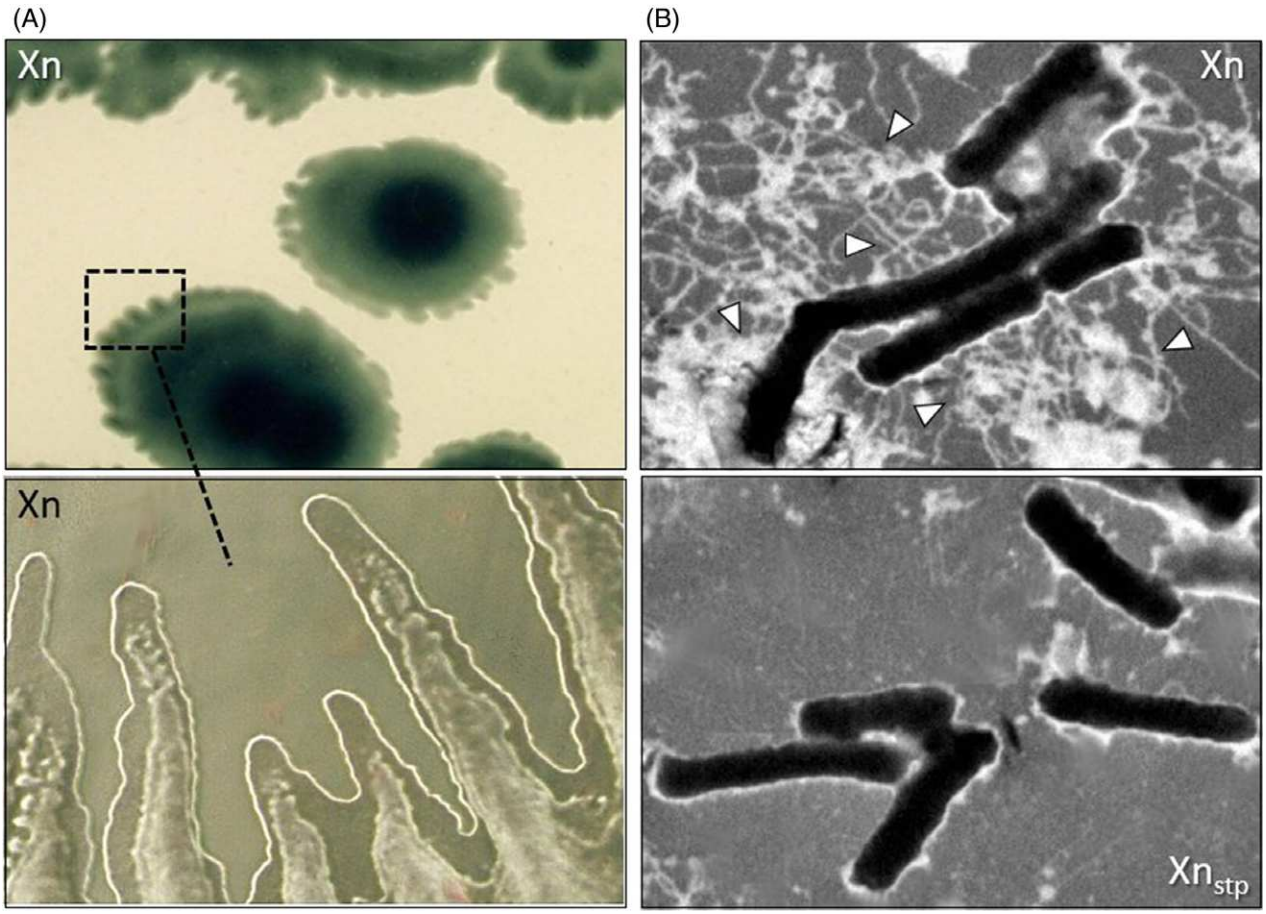

Figure 2. Assessment of *X. nematophila* phase I bacterial morphology and evidence of structural changes after high-salt treatment. (A) Morphology of blue colonies of *X. nematophila* bacteria grown on soft NBTA and plated on 0.8% agar. Phase I bacterial colonies (Xn) show the characteristic swarming behaviour observable in the peripheral area of the colony; the image below shows a magnification of the boundary of colonies from the image above acquired by light microscopy. (B) Transmission electron microscopy micrographs of untreated and high-salt treated (stripped) *X. nematophila* after negative staining. The image above (Xn) shows the typical phenotype of phase I bacteria: the presence of surface structures is evident, such as pili/fimbriae and flagella (arrowheads) that protrude extending outward from the bacterial surface. The micrograph below (Xn_{stp}) shows the absence of the structures removed by high-salt treatment (stripping) from the bacterial surface. Xn, *X. nematophila* phase I bacteria; Xn_{stp}, *X. nematophila* bacteria stripped after high-salt treatment.

3 RESULTS

3.1 Analytical electrophoresis of *X. nematophila* and *S. carpocapsae* surface proteins

To analyse the protein pool in samples eluted by high-salt treatments, either from bacterial cell walls (XoP) or from parasite cuticles (SoP), we carried out analytical electrophoresis separations (Fig. 1). The XoP electrophoretic pattern for live bacteria (Fig. 1; XoP_L) showed three major bands of 17, 36 and 46 kDa, and some minor bands were also observable. The XoP pattern for heat-killed bacteria (Fig. 1; XoP_D) was comparable to that for live bacteria, apart from some quantitative differences. The protein pool eluted from the surface of both live and dead parasites (Fig. 1; SoP_D and SoP_L) revealed two major bands of 54 and 69 kDa, and some minor bands.

3.2 Bacterial and colony morphology

The morphology of phase I *X. nematophila* plated on soft NBTA was observed using a stereomicroscope or light microscope and, as expected, the colonies showed typical swarming behaviour (Fig. 2A; Xn, top and bottom images). The lack of motility structures, such as flagella, was further confirmed by the loss of swimming ability of stripped bacteria observed in liquid medium by light microscopy (data not shown).

The presence of outer structures on the cell wall of *X. nematophila* was examined by TEM. After negative staining (Fig. 2B; Xn, top), the presence of filamentous structures (arrowheads), common in phase I symbionts, was evident, whereas salt treatment almost completely removed fimbriae and flagella (Fig. 2B; Xn_{str}, bottom).

3.3 Cytotoxic properties of nematocomplexes and their compounds on *G. mellonella* haemocytes

To determine whether surface molecules or secretions from live or dead *X. nematophila* or live or dead *S. carpocapsae* affected the vitality of host haemocytes, we performed MTT viability assays.

The two-way ANOVA results showed a significant difference between the different conditions (i.e., controls and different treatments) tested in the assays carried out with *X. nematophila* ($df = 6$, $F = 1687$, $p < 0.0001$; Figs. 3 and 4) and in those carried out with *S. carpocapsae* ($df = 4$, $F = 587$, $p < 0.0001$; Fig. 5). All the differences between treatments and corresponding controls and among treatments were statistically significant (Tukey HSD test,

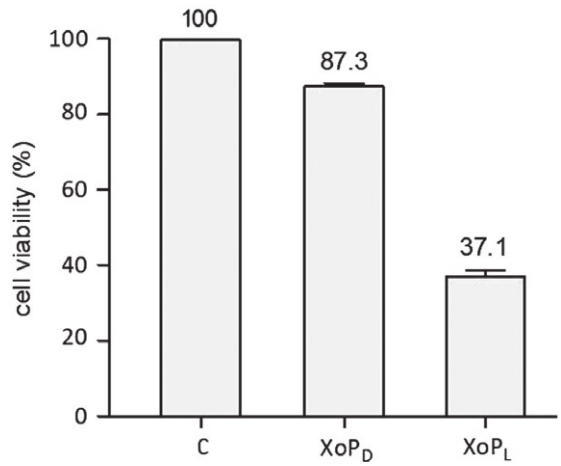

Figure 3. Viability test on XoP-treated host haemocytes, and MTT test on cultured haemocytes from *G. mellonella* in the presence of XoP eluted from dead (XoP_D) or live (XoP_L) phase I *X. nematophila*. XoP cytotoxic effects were evident when cells were treated with 150 ng/μl of XoP from live bacteria (XoP_L); in contrast, when XoP were extracted from killed bacteria (XoP_D), a lower effect was observable. All treatments were carried out for 24 h. The results are expressed as the percentage of cell viability observed in different treatment conditions and the respective standard deviation compared with control cultures (C), which were considered to have a value of 100%. XoP_D, haemocytes incubated with outer proteins from dead *X. nematophila* bacteria; XoP_L, haemocytes incubated with outer proteins from live *X. nematophila* bacteria; C, control haemocytes.

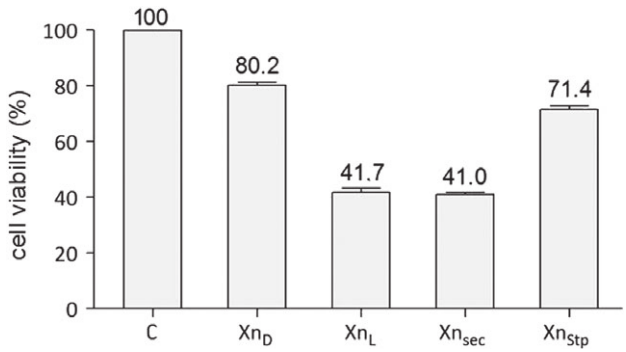

Figure 4. Viability test on *X. nematophila* (Xn)-treated host haemocytes. Cultured haemocytes were treated with dead (Xn_D) and live (Xn_L) *X. nematophila*. Cell viability was also assayed in the presence of bacterial secretions (Xn_{sec}) and with bacteria without XoP (Xn_{stp}). Live Xn and its secretions showed a marked cytotoxic effect on host haemocytes. Cell mortality was strongly reduced when haemocytes were incubated with Xn_{stp} bacteria. All treatments were carried out for 24 h. The results are expressed as the percentage of cell viability observed in different treatment conditions and the respective standard deviation compared with control cultures (C), which were considered to have a value of 100%. Xn_D, haemocytes incubated with outer proteins from dead Xn bacteria; Xn_L, haemocytes incubated with outer proteins from live Xn bacteria; Xn_{sec}, haemocytes incubated with Xn secretions; Xn_{stp}, haemocytes incubated with Xn stripped after high-salt treatment; C, control haemocytes.

$P \leq 0.001$) except for live *X. nematophila* (Fig. 4; Xn_L) and their secretions (Fig. 4; Xn_{sec}) which induced the same effect.

The cytotoxic effect of XoP when extracted from live *X. nematophila* was evident: at 150 ng/μl, XoP induced >60% mortality of host cells (Fig. 3; XoP_L). This effect was drastically reduced when the host cells were treated with XoP derived from heat-killed bacteria (Fig. 3; XoP_D). Although statistically different, the average toxicity level of XoP_L (Fig. 3) was similar to those of live

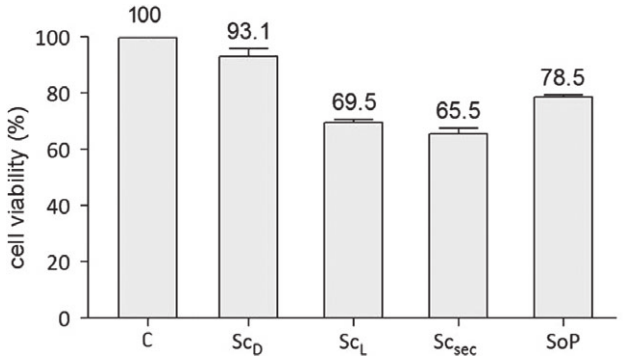

Figure 5. Haemocyte viability test in the presence of *S. carpocapsae*. Assays were carried out with cultured host cells in the presence of dead (Sc_D) and live (Sc_L) parasites, parasite secretions (Sc_{sec}) and their surface protein components (SoP). Live parasites (Sc_L) and SoP induced about 30% and 20% haemocyte mortality, respectively. Effects caused by parasite secretions (Sc_{sec}) were comparable to the mortality observed with live nematodes (about 35%). Dead nematodes (Sc_D) induced just 7% mortality. All treatments were carried out for 24 h. The results are expressed as the percentage of cell viability observed in different treatment conditions and the respective standard deviation compared with control cultures (C), which were considered to have a value of 100%. Sc_D, haemocytes incubated with dead *S. carpocapsae* nematodes; Sc_L, haemocytes incubated with live *S. carpocapsae* nematodes; Sc_{sec}, haemocytes incubated with secretions from *S. carpocapsae* nematodes; SoP, haemocytes incubated with outer proteins from live *S. carpocapsae* nematodes; C, control haemocytes.

X. nematophila (Fig. 4; Xn_L) and their secretions (Fig. 4; Xn_{sec}). As for XoP_D, the cytotoxic properties showed by symbiotic bacteria were lost if they were deprived of XoP surface molecules (Xn_{str}) or when haemocytes were incubated with dead bacteria (Xn_D).

Also, live parasites (Fig. 5; Sc_L) and their secretions (Sc_{sec}) showed a cytotoxic effect. However, the average induced mortalities were approximately half of those caused by isolated symbiotic bacteria (Fig. 4). A significantly lower toxicity was observed in the presence of both SoP (Fig. 5), causing approximately 20% mortality of haemocytes, and dead parasites (Fig. 5; Sc_D), causing <10% mortality.

3.4 Phagocytosis of host haemocytes in the presence of XoP or XoP-deprived *X. nematophila*

To evaluate haemocyte phagocytosis, we used *S. aureus* labelled with pHrodo fluorescent probe, as a target, following an injection of outer protein samples from live *X. nematophila* (Fig. 6; XoP) or of stripped *X. nematophila* (Fig. 7; Xn_{str}). The two-way ANOVA results showed a significant difference between controls and treatments tested in the case of both XoP assays ($df = 1$, $F = 387$, $p < 0.0001$; Fig. 6) and Xn_{str} assays ($df = 1$, $F = 14$, $p < 0.001$; Fig. 7). A significant reduction (Tukey HSD test; $P < 0.0001$) of phagocytosis by host cells when larvae were previously injected with XoP was observed (Fig. 6; XoP): the average phagocytosis percentage was almost half that of the control (Fig. 6; C). Although the difference from the control was statistically significant (Tukey HSD test; $P < 0.001$), the removal of the outer compounds from the bacterial surface (Fig. 7; Xn_{str}) resulted in a marked loss of inhibitory properties.

3.5 Effects of live parasites on host phagocytosis

The ability of host haemocytes to phagocytose was assessed after the injection of the nematocomplex *S. carpocapsae* (Fig. 8A and B). The presence of nematodes a short time following infection (< 1 h) did not significantly modify the phagocytosis level (Fig. 8A; Sc_{1h}). However, a significant effect (two-way ANOVA for variable

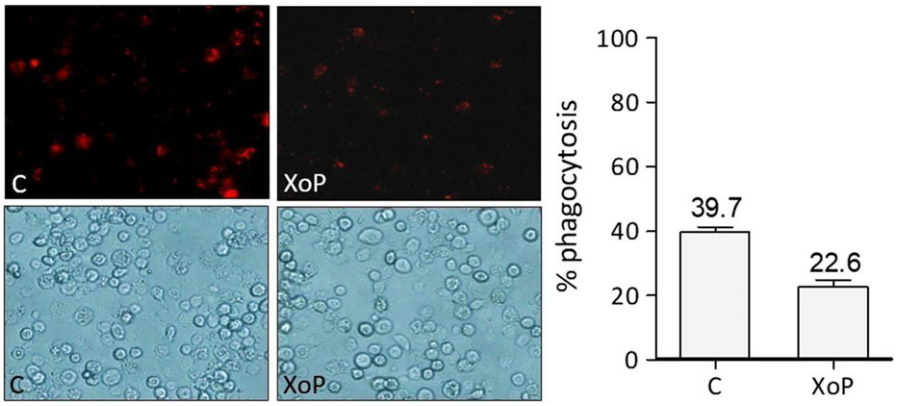

Figure 6. Effects of XoP on haemocyte phagocytosis activity. Larvae were injected with XoP and then with pHrodo-conjugated *S. aureus*; haemocyte phagocytosis of *S. aureus* was evaluated by epifluorescence microscopy. The micrograph in the upper left panel (C) shows the bright emission of phagocytosed *S. aureus* cells (control assay); in the upper right panel (XoP-injected larvae), a marked decrease of phagocytosis can be observed. Histograms on the right show the percentage of phagocytosis of control haemocytes (C) and of haemocytes from XoP-injected larvae; the presence of XoP decreased by half the host phagocytosis capability. The lower micrographs are the bright field views of the upper fluorescence images. C, control; larvae injected with PBS and after 1 h with *S. aureus*-pHrodo; XoP, larvae injected with *X. nematophila* surface proteins and after 1 h with *S. aureus*-pHrodo.

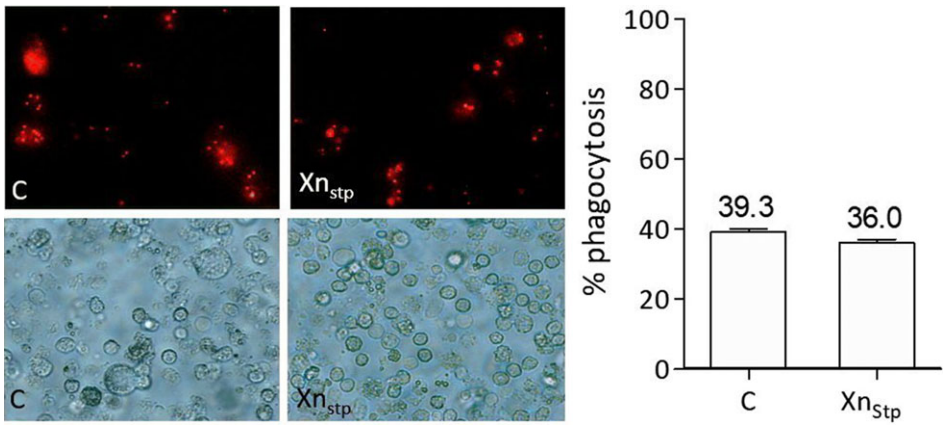

Figure 7. Effects of the injection of stripped *X. nematophila* (Xn_{stp}) on the phagocytosis activity of *G. mellonella* haemocytes; pHrodo-conjugated *S. aureus* was injected 1 h after Xn_{stp}. Haemocyte phagocytosis was not markedly affected by the presence of *X. nematophila* lacking XoP. Histograms on the right show the percentage of phagocytosis of control (C) and Xn_{stp}-treated haemocyte cultures. The lower micrographs are the bright field views of the upper fluorescence images. C, control; larvae injected with PBS and after 1 h with *S. aureus*-pHrodo; Xn_{stp}, larvae injected with stripped Xn and after 1 h with *S. aureus*-pHrodo.

'condition': df = 1; $F = 652$; Tukey HSD test; $P < 0.0001$) was observed when parasite infection was protracted to 3 h (Fig. 8B; Sc_{3h}); 3 hours after infection the decrease in phagocytic activity was about 20% and this result is comparable to that achieved with the injection of XoP (Fig. 6; XoP).

3.6 proPO system modulation by XoP and live *X. nematophila*

We analysed *in vitro* phenoloxidase activity in haemolymph from *G. mellonella* larvae in the presence of XoP and live *X. nematophila*. The time-course of dopachrome formation in Fig. 9 (left) shows that the XoP did not inhibit the host phenoloxidase, and thus they seem to strongly trigger the host proPO system activity. In contrast, marked inhibition was observed when haemolymph was co-incubated with live bacteria (Fig. 9; right).

4 DISCUSSION

In this study, we investigated the immunomodulatory role of high salt-soluble components isolated from the surface of *S. carpocapsae* and from its symbiotic bacterium, *X. nematophila,* in the immune response of the host *G. mellonella*.

Current literature on the parasitic strategies of EPNs suggests a synergistic cooperation between the nematode and its bacteria, in which the parasite seems to rapidly implement an elusive approach, required to escape recognition by its host, before the release of its bacterial symbionts in the mid phase of the infection.

Many works have been focused on the compounds secreted from bacteria or nematodes and their capability to drastically alter host physiology. In these works, many authors recognised the symbionts to be primarily responsible for the death of the host.[36–38] Thus, in the EPN complex, nematodes and bacteria work together to alter the physiological balance of the host, primarily affecting its immunological competence, leading it to be particularly susceptible to septicaemia, which occurs in the late infection phase.

After parasite intrusion, host immune receptors first interact with molecules present on the surface of the foreign body. This interaction can lead to different responses, such as lack of recognition (molecular disguise or mimicry) or active immune

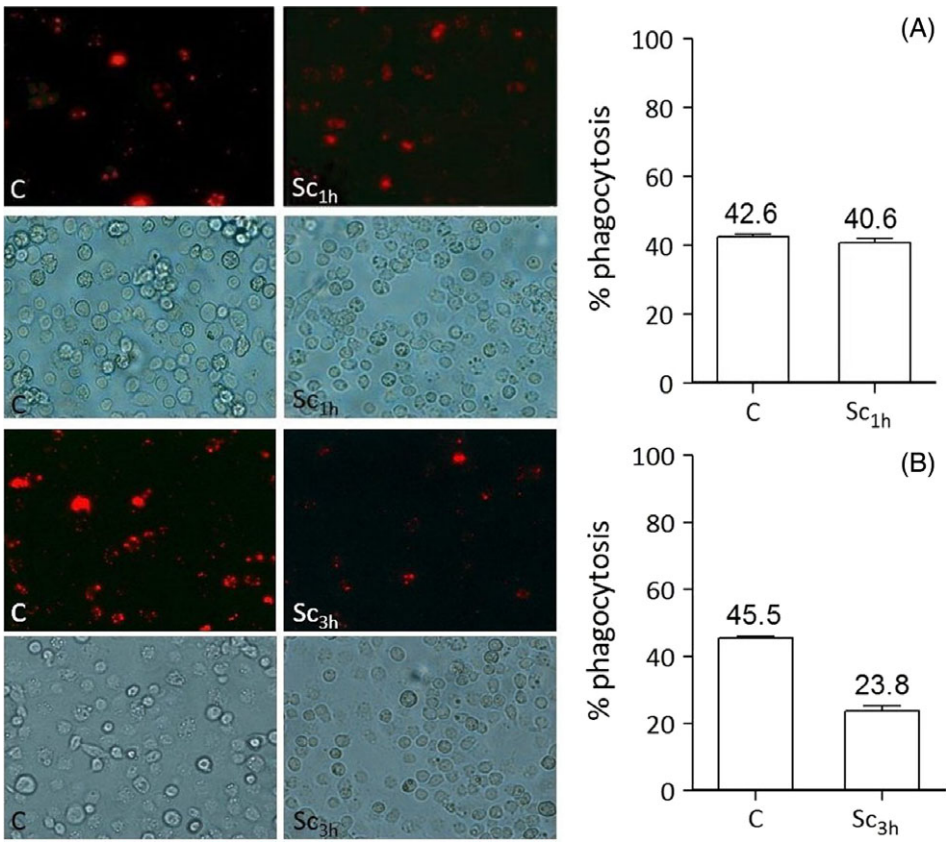

Figure 8. Effects of the injection of live *S. carpocapsae* on haemocyte phagocytosis activity in *G. mellonella* larvae. Larvae were infected with *S. carpocapsae* (Sc) and, 1 or 3 h later, pHrodo-conjugated *S. aureus* was injected. *Staphylococcus aureus* phagocytosis was not significantly affected by 1 h of the presence of parasites (A; Sc_{1h}), and the phagocytosis level was comparable to that of control assays (C). The prolonged presence (3 h) of parasites (B; Sc_{3h}) resulted in a marked decrease of phagocytosis activity. The lower micrographs are the bright field views of the upper fluorescence images. Histograms on the right show the percentage of phagocytosis of control (C) and Sc_{1h-3h}-treated haemocyte cultures. C, control; larvae injected with PBS and after 1 h with *S. aureus*-pHrodo; Sc_{1h-3h}, larvae injected with live Sc and after 1 or 3 h with *S. aureus*-pHrodo.

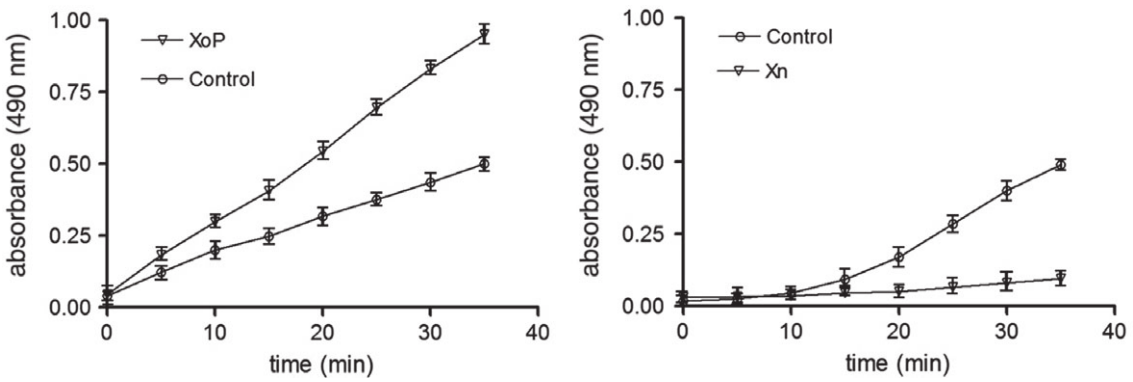

Figure 9. Host proPO system modulation in the presence of live *X. nematophila* (left) or its surface proteins (XoP) (right). The presence of *X. nematophila* induced a marked inhibition of the host proPO system (left; Xn). In contrast, the bacterial surface compounds (XoP) strongly activated the proPO system (right; XoP). Values are averages ± standard deviation.

depression of the host.[3,39–43] Both processes have been well described in *Steinernema feltiae*, and it has been demonstrated that the cuticle/epicuticle of this EPN, particularly its lipid moieties, actively interferes with both humoral and cellular responses of *G. mellonella* larvae.[14,20,22,40,44]

A recent paper[45] suggests that *S. carpocapsae* or *H. bacteriophora* cuticles affect several immunological processes of *G. mellonella* larvae; purified whole cuticles interfere with haemocyte vitality and phagocytosis, phenoloxidase activity and antimicrobial activity.

In a previous study, we demonstrated that the cuticle of *S. carpocapsae*, when injected into *Rynchophorus ferrugineus* larvae, is mainly responsible for elusion of cell surveillance, although host haemocytes were not damaged by the presence of the cuticle.[46] Likewise, the parasite cuticle did not activate or inhibit the host proPO system, and comparable results were also obtained with dead nematodes. Instead, phenoloxidase activity was decreased in the presence of live nematodes (30 min after infection), or a marked activation was evident in the mid phase of infection,

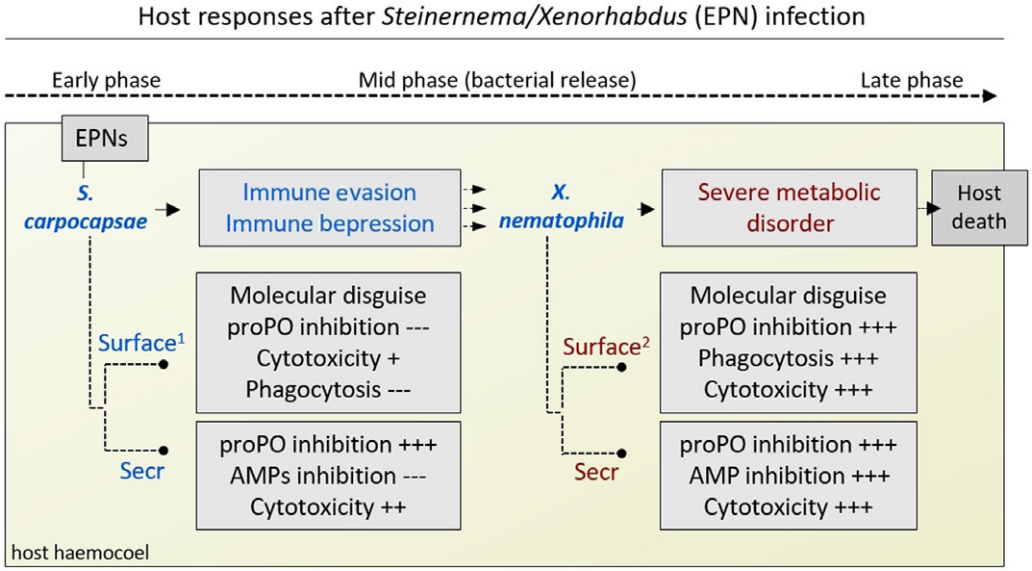

Figure 10. Summary scheme of the main effects induced by parasites, symbionts and their secretions and surface compounds.

probably attributable to the parasite's secretions and the presence of *X. nematophila* released in the haemolymph.

In this work, we isolated and analysed a protein pool derived from *S. carpocapsae* cuticle/epicuticle elution (SoP); the electrophoretic pattern showed few bands with molecular masses comparable to the surface coat proteins (SCPs) described by Li *et al.*[40] We observed that SoP did not interfere with host cell activity and viability. Moreover, when host larvae were infected with whole *S. carpocapsae*, haemocyte phagocytosis was not affected, because hemocytes phagocytized *S. aureus* as the cells obtained from naïve larvae.

However, after several hours of treatment with the SoP of *S. carpocapsae*, low mortality of the host cells was detected during cytotoxicity assays. As expected, a significant effect was observed when haemocytes were incubated with live parasites or their secretions. Our data agree with the findings of various authors that confirmed that the immunomodulatory role of the nematode was attributable to its secretions, which culminates in interference with the host's immune processes.[3,7] The different behaviour observed by Yi *et al.*[45] could be explained by the fact that their assays were carried out with whole cuticles, unlike in this work, where the observed effects were exclusively attributable to the salt-soluble protein moiety of the parasite body surface.

In addition to the study of the nematode surface, we have investigated the immune-modulating properties of protein components from surface structures of the bacterial wall of phase I *X. nematophila*. Firstly, we have ascertained the relevant phase of the symbionts by means of selective culture methods and by examining the morphology of the bacteria. Electron microscopy ultrastructure, the swarming and swimming capability of colonies, dye adsorption and the presence of pili/fimbriae and flagella were the main criteria used to distinguish the relevant phase of the symbionts. With the same criteria, we have verified the removal of *X. nematophila* surface structures in stripped bacteria after high-salt treatment.

The elution of soluble proteins from *X. nematophila* (XoP) showed a pattern of bands ranging from 17 to 46 kDa when analysed by SDS-PAGE. According to the current literature, the 17-kDa band could be identified as pilin, a major constituent of bacterial pili/fimbriae[24]; the band at approximately 36 kDa could be flagellin, the major component of bacterial flagella[47]; and further proteic bands could derive from the outer membrane vesicles of the bacteria.[26] The decrease of the band intensity at 36 kDa in XoP_D samples could be attributable to partial detachment of flagella during heat treatments carried out to kill bacteria.

All the eluted proteins from EPN complexes are components of supramolecular structures whose immunological role is well known. Generally, bacterial OMV, fimbriae, flagella and the nematode cuticle/epicuticle are responsible for immunosuppression and/or immunoevasion of the host.[3,21,23,39,45,48]

Bacterial XoP, derived from live symbionts, showed a marked cytolytic activity on *G. mellonella* haemocytes. The observed effects resulted in a mortality rate comparable to that induced by *X. nematophila* secretions or by the live bacteria. Various processes of injury to host haemocytes caused by *X. nematophila* have long been described.[49]

A marked decrease of cytotoxicity was observed when haemocytes were incubated with XoP-deprived (stripped) bacteria. The reduced effects of symbionts lacking XoP further confirmed the role played by surface proteins.

Moreover, slight cytolytic effects were detected in the presence of heat-killed *X. nematophila* and its XoP. The limited activity was probably attributable to partial protein denaturation, which disarranges the molecular architecture of the outer structures.

When *G. mellonella* larvae were infected with fluorescent-conjugated *S. aureus*, haemocytes strongly reacted and about 50% of immunocompetent cells engulfed bacteria. As previously described, in the early period after infection (within 1 h), live *S. carpocapsae* did not interfere with host phagocytosis; instead, a marked inhibitory effect was evident when symbionts were released in the mid phase.

Prior injection of XoP obtained from live symbionts induced a significant decrease in the phagocytic activity of the haemocytes: a reduction to approximately one half when compared with control assays. In viability tests, we observed a moderate cytotoxic activity of XoP, so it is reasonable to suppose that the decreased levels of phagocytosis could be attributable to both cell damage and the lack of recognition of non-self.

Moreover, the effects of *X. nematophila* surface proteins were further confirmed by the lack of inhibition observed when *G. mellonella* larvae were pre-injected with stripped bacteria. In this case, the level of phagocytosis was comparable to that of controls.

In the initial phase of infection, the EPN complexes need to impair early defensive processes, such as phenoloxidase activity. This action seems to be mediated mainly by nematode secretions, or later, by symbiont metabolites and peripheral small molecules such as rhabduscin.[9]

In this work, we observed that, when proPO system activity was assayed *in vitro*, the proteic pool from the *X. nematophila* surface activated the haemolymph prophenoloxidase, but, even if this action seems to be self-defeating, it could be counteracted *in vivo* by the presence of rhabduscin, which is inhibitory at nanomolar concentrations,[50] and subsequently by the effect of various secreted factors also responsible for inhibition of the host proPO system. The data obtained with live *X. nematophila* showed the negative modulation of the host proPO system, as a result of the inhibitory properties of various compounds secreted by the symbionts.

During parasitism, we can observe situations of shared benefit, including mutualism, and extreme conditions, such as those involving parasitoids capable of killing their hosts. The latter is the case for EPN, which establishes a tripartite relationship with its symbiotic bacteria and the target insect host. The complexity of EPN parasitism and its importance in pest control techniques have led to many studies to obtain a deeper understanding of the mechanisms by which entomopathogen complexes overcome the host immune system. The complexity of EPN–host relationships is further complicated by the presence of bacterial symbionts and by the spatio-temporal events that occur after host infection. A few minutes after entry, the nematode alone must fight against the early immune defences of the host, so its strategies of elusion or depression are essential for survival and to create a favourable environment before the release of symbionts. Also, its symbionts are delivered into a potentially harmful internal *milieu* before they can use their toxic secretions, so the earlier interactions involve its cell wall surface, which can actively support the bacteria to achieve their deadly goal.

The complexity of the events observed within the phases of EPN infection is summarised in (Fig. 10). The graphical representation highlights the main effects induced by parasites, symbionts, secretions and surface compounds.

The results of this study are focused on the overall effects induced by a pool of proteins derived from the surface of parasites and bacteria, and they represent a starting point for specific characterisation of the single protein components that are responsible for the observed immune depressive effects.

Many works have added significantly to our understanding of host–parasite relationships; several factors, either from nematodes or from their symbiotic microorganisms, have been identified as participants in such interactions between EPNs and their target insects. Most of them are released from EPNs by secretory processes, although information about the roles of surface proteins is still scarce.

The development of new biological control strategies is aimed to improve the effectiveness of bio insecticides, such as bacteria, fungi or nematocomplexes. Many studies have focused on the effects of secretions/metabolites produced by these organisms, instead our study was focused on investigating the immunosuppressive role of molecules present on the body surface of these organisms, in view of their importance in the earliest interaction with the host body. The success of entomopathogens is mainly reliant on avoidance of immune recognition in the early phases of the parasitisation, a brief time interval in which the host immune system discriminates the invader's body surface as self or not self, thus triggering or not further self-protecting processes. We believe that increasing knowledge of insect host–parasite relationships will greatly improve our understanding of non-self-recognition processes that are typical of insect immunity and could be a good starting point in improving the biological control of insect species potentially harmful for urban green and agriculture.

ACKNOWLEDGEMENTS

We are grateful to Dr Michela Curradi for critical reading of the manuscript and to Dr Silvia Quadroni for statistical analysis. This work was supported by FAR funds from the University of Insubria.

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CHAPTER 4

The influence of climate change on the host-parasite relationships

4.1 Introduction

According to the fourth report of the Intergovernmental Panel on Climate Change (IPCC, 2014), the average temperature of the earth's surface increased by 0.74 ± 0.18 °C during the 20th century: this process is inducing a change in the earth's climate. The increase in temperatures is due to the emission of greenhouse gases into the atmosphere (CO_2, CH_4, O_3, H_2O) and has been observed since the second industrial revolution (fig. 19).

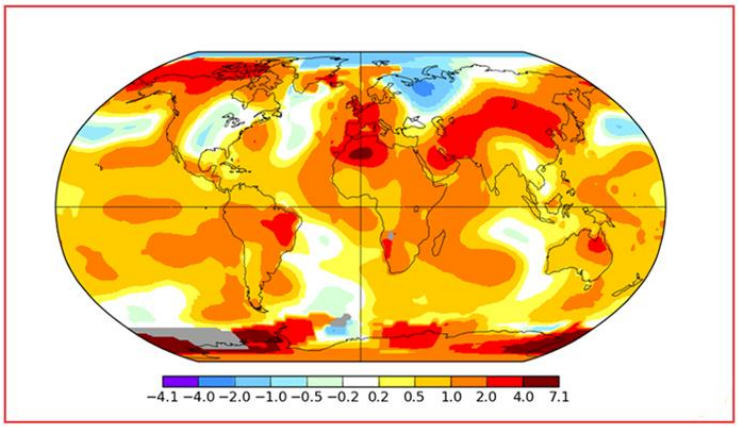

Fig. 19 - Global temperature increase from 1951 to 1980.

The environmental temperature is considered one of the major factors of ecological impact that affects the physiology of ectothermic organisms, the ability to tolerate temperature fluctuations is essential for survival. As a result, global warming can pose a substantial challenge to many species, and to those ectothermic organisms that live near their thermal limits, making them particularly vulnerable to temperature changes (Grewal et al, 1994 - Hazir et al, 2001).

Insects are one of the groups of organisms particularly sensitive to climate change because temperature has a direct influence on their development, reproduction and survival. Various Insects species have a well-defined life cycle divided in various stages (larva, pupa, adult), many of them have more than one generation in the same year; for this reason, they can represent excellent bioindicators in terms of speed of adaptation compared to long-lived organisms.

Atmospheric warm-up can heavily affect insect physiology, and potential responses may also involve changes in phenological patterns, habitat choice or expansions/contractions in geographical and altitude ranges (Hopper et al, 1973). Another important risk related to the

change in temperature, may be the colonization of unusual habitats by harmful insects, this could result in the colonization of areas where the natural competitors of that species might not be present. This colonization may also concern species considered beneficial. Indeed, if their development in unusual habitats is also excessive, it can damage the environment themselves even though they are not defined as harmful species in general.

Given the importance of insects' populations, both for health and for human activities such as agriculture, many researchers have focused their attention on insects in temperate regions; global warming can also benefit many species of insects, the decrease in the duration of cold months can be favorable to survival and increase in insect populations. Global warming can extend seasonal development by increasing growth, reproductive rate and number of annual generations. Areal changes, together with physiological alterations, could lead to severe consequences, the lack of natural predators and competitors in the newly colonized areas, as well as a possible greater resistance of migrant species. The absence of effective natural enemies in the new habitat and a greater plasticity of the invasive species (compared to the native ones), can increase the spread of the invasive species with a damage to the ecosystem.

The general consequences of these phenomena affect both the impact on agricultural production and on urban greenery by phytophagous species, but also a health warning for the spread of diseases transmitted by non-native vectors (Pachauri et al, 2007 – Björkman et al, 2015).

4.2 Effects of temperature changes on insects

One of the main effects of global warming is the physiological adaptation and expansion of the areas of many species called "harmful" insects; indeed, being ectothermic they are not able to regulate their body temperature, for this reason their biological cycle and their distribution is strongly influenced by environmental parameters (Bebber et al, 2014). These aspects can have dramatically important implications when we refer to species that are harmful to crops (phytophagous) or to health (vectors of diseases).

For example, in the Italian Alps in the period from 1974 to 2004, the pine processionary (*Thaumetopoea pityocampa*), a dangerous parasite of many conifers, has extended its effects areal to higher altitudes, causing serious damage to the local flora (Hódar et al, 2003). Since 2012, the Asian bug (*Halyomorpha halys*) has been found in some Italian regions, causing serious damage to

fruit crops, vineyards and vegetables. Among the vectors of disease, the genus *Phlebotomus* responsible for leishmaniasis, is currently expanding in the Italian continental areas. A further problem, confirmed in recent years, is the spread of viral diseases and typically tropical fevers such as Chikungunya, which appeared in Italy following the spread of vector insects such as *Aedes albopictus*, known as tiger mosquito, capable of carrying at least twenty different types of viral diseases (Reinhold et al, 2018). The appearance of diseases, transmitted by non-native vectors, can derive from climate change, but is also related to socio-economic factors and international trade.

These biological and environmental emergencies require a deep review of biological control methods with bio-insecticides, since we expect that climate change can also have an impact on biological control agents. In general, nematodes, bacteria and fungi used as biopesticides are vulnerable to environmental stress (temperature, humidity), which can result in physiological changes. It is therefore necessary to review the use of some bio-insecticides on their usual targets and on any new alien species (Denlinger et al, 1998 - Chaves et al, 2012).

4.3 Paper summary. *Susceptibility to entomopathogens and modulation of basal immunity in two insect models at different temperatures.*

We planned to evaluate the effectiveness of bio-insecticides by varying parameter such as environmental temperature, the assays were carried out by protocols (white trap method) that simulate what happens under usual field conditions.

4.3.1 Introduction

In literature there are several studies concerning the effectiveness of entomopathogenic organisms such as EPN or *Bacillus thuringensis* (for a review see: Brivio et al, 2018), but research on the effects of global warming as a consequence of the climate change, are still relatively scarce. For this reason, in the paper presented below we focused on the role of temperature as a parameter that can affect the parasite's efficacy and the subsequent host response.

We compared, the action of three different EPNs (*S. feltiae*, *S. carpocapsae* and *H. bacteriophora*) and of *Bt* against the greater wax moth, *Galleria mellonella* (Lepidoptera) and against the fly *Sarcophaga africa* (Diptera). These two insect models are known respectively as honeybee pest (*G. mellonella*) and vector of diseases both in human and animal (*S. africa*).

We also investigated the modulation of the basal humoral immunity of the insects' host under different rearing temperature. In particular, considering the important role of the insect proPO system in the host immune responses and the bacterial clearance carried out by the activity of lysozyme, we evaluated the influence of the temperature on the PO and lysozyme activity in the host hemolymph.

4.3.2 Materials and methods

EPNs were stored at 4 °C to keep parasites in cryptobiosis and *Bt* was supplied as a suspension. *G. mellonella* and *S. africa* were fed with sugar and water ad libitum and kept under controlled conditions. As natural infestation method, we used the *white trap* method to evaluate the lethality of both the three EPNs and *Bt* against the insect models.

Insects reared under normal conditions were then placed in climatic chambers at the established temperatures (10, 20, 30 °C) with constant relative humidity (70 ± 10%). The mortality rate due to the effects of the bioinsecticides was evaluated at intervals of 12 h, for 60 h. Three replicates were carried out for each bio-insecticide and temperature investigated.

The amount of insect hemolymph (30 µL/larvae) was collected by puncturing prolegs of last instar larvae. Whole hemolymph samples were processed by two low-speed centrifugations to remove cells and tissue debris and the CFF (cell-free fractions) were immediately assayed for enzymatic activities.

The PO activity was evaluated by spectrophotometric analysis of CFF samples and the enzymatic assay of lysozyme was carried out by turbidimetric assays with a suspension of *M. luteus* bacteria (for details see the paper below).

4.3.3 Summary of results

The susceptibility of *G. mellonella* to the EPN *S. feltiae* is evident mainly at the temperature of 20 °C, after 60 h, when the mortality rate reaches the 100%. The Diptera *S. africa* was instead less affected by the same EPN: the mortality rate never exceeded (at any temperature) the 50% of mortality on average. The other EPN species (*S. carpocapsae*) was significantly more lethal at the highest temperature tested for both the insects' models.

The third EPN investigated (*H. bacteriophora*) had a behavior like *S. carpocapsae*: at 30 °C after 60 h of exposure, the mortality of both insects was over 80% on average. About the assays with *Bt*, they show a significantly higher efficacy at 10° and 20 °C for *G. mellonella* and at 30 °C for *S. africa*.

The PO activity in *G. mellonella* was significantly higher at 30 °C and the relative activity of the hemolymph lysozyme of *G. mellonella* significantly increases with the temperature. On the contrary, *S. africa* possesses a negligible lytic activity at any temperature tested (tab.3).

	10 °C				20 °C				30 °C			
	Sf	*Sc*	*Hb*	*Bt*	*Sf*	*Sc*	*Hb*	*Bt*	*Sf*	*Sc*	*Hb*	*Bt*
Gm	+	–	+	+++	+	++	+	+++	–	+++	++	–
Sa	–	–	+	–	–	–	+	–	+	+	++	++

−: Mortality < 25%; +: 25 ≤ Mortality ≤ 50%; ++: 50 ≤ Mortality ≤ 75%; +++ : Mortality > 75%.

Tab 3. Cumulative table of the efficacy (referred to 48 h post administration) of S. feltiae (Sf), S. carpocapsae (Sc), H. bacteriophora (Hb) and B. thuringensis (Bt) on G. mellonella (Gm) and S. africa (Sa), after conditioning at 10, 20 and 30°C.

In conclusion, our data suggest that the relationships between entomopathogens and their hosts could be altered by the temperature changes, thus affecting the efficacy of the bio-insecticides on the target insects in specific ways in different species. Although the enhancement of immune activity does not seem to be the main factor responsible for an increased resistance to entomopathogens, the temperature variations can influence the host immune response. So, both the success of a bio-insecticide and the insect survival can differ because of environmental variations, which could be responsible for physiological changes of the host and the entomopathogens. Even though we are aware that further studies are required to deepen the relationships between entomopathogens and their hosts in the climate change context, we believe that our work may provide a valuable starting point to investigate the effects of temperature variations on the host-parasite relationships and biological control methods.

Journal of Thermal Biology 79 (2019) 15–23

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

Susceptibility to entomopathogens and modulation of basal immunity in two insect models at different temperatures

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ARTICLE INFO

Keywords:
Insect immunity
Entomopathogen nematodes
Bacillus thuringiensis
Temperature

ABSTRACT

In this work, we analysed the efficacy of different commercial bio-insecticides (*Steinernema feltiae, Steinernema carpocapsae, Heterorhabditis bacteriophora* and *Bacillus thuringiensis*) by valuating the mortality induced on two insect models, *Galleria mellonella* (Lepidoptera) and *Sarcophaga africa* (Diptera) after exposure to different temperatures (10, 20 and 30 °C). Moreover, we investigated the effects of temperature on the basal humoral immunity of the two target insects; particularly, phenoloxidase (PO) and lysozyme activity. Our results show that *G. mellonella* is susceptible to all bio-insecticides at all the examined temperatures, except when infected at 10 °C with *S. carpocapsae* and at 30 °C with *S. feltiae* and *B. thuringiensis. S. africa* is more susceptible at 30 °C to all bioinsecticides; whereas, when infected at 10 and 20 °C, *H. bacteriophora* is the most efficient. Temperature modulates PO activity of both *G. mellonella* and *S. africa*, otherwise variations in lysozyme activity is observed only in *G. mellonella*. Except for a possible correlation between the increased lysozyme activity and the delayed *Bt* efficacy recorded on *G. mellonella* at 30 °C, a different resistance to bio-insecticides at different temperatures does not seem to be associated to variations of the host basal immunity, probably due to immunoevasive and immunodepressive strategies of these entomopathogens.

1. Introduction

In recent years, a large expansion of the home range of many species of so-called harmful insects has been observed (Lindgren et al., 2012; Aguilar-Fenollosa and Jacas, 2013; Baylis, 2017). The main cause of this worldwide phenomenon has been ascribed to changing climatic conditions (Abram et al., 2017). In particular, the modification of insects' distribution in latitude and elevation and in the timing of their life cycles is related to long-term, annual variations in temperature (Chen et al., 2011). These changes would easily lead to an uncontrolled spread of pests (i.e., phytophagous and vectors species) in different areas around the world where few enemies and natural competitors are present (Okulewicz, 2017; Renault et al., 2018).

According to the current integrated pest management practices (Chandler et al., 2011; Barratt et al., 2018), any action aimed to control the increased diffusion of vectors or phytophagous insects should not increase the use of synthetic chemical pesticides due to their well-known adverse impacts. Otherwise, environmentally-friendly methods based on the use of bio-insecticides such as predatory insects and mites, parasitoids, parasites and microbial pathogens should be intensified. However, like their target insects, these organisms are susceptible to variations in the environmental temperature. It is thus necessary to reconsider the physical conditions in which bio-insecticides are highly effective.

Among the most widely used bio-insecticides are entomopathogenic nematodes (EPNs) and *Bacillus thuringiensis* (*Bt*). EPNs are insect endoparasites belonging to the genera *Steinernema* (Rhabditidae, Steinernematidae) and *Heterorhabditis* (Rhabditidae, Heterorhabditidae). They are commercially available as inundative control agents against a variety of pests (Shapiro-Ilan et al., 2006; Lacey et al., 2015). These nematodes are mutualistically associated to bacteria of the genera *Xenorhabdus* and *Photorhabdus,* respectively (Eleftherianos et al., 2010). The combined action of both worms and bacteria results in the elusion from host immune surveillance and in a marked immune depression that culminates in the death of the target insect (Castillo et al., 2011; Eleftherianos et al., 2017). Castillo et al. (2011) have described the mechanisms by which the nematode and its symbionts interfere with the immunological processes of recognition and elimination of non-self, triggered by the host when in the presence of foreign invaders. Since overcoming the host defences is an essential prerequisite for a successful infection/parasitisation, many entomopathogens have evolved strategies to bypass host receptor-mediated immune

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https://doi.org/10.1016/j.jtherbio.2018.11.006
Received 7 June 2018; Received in revised form 31 October 2018; Accepted 12 November 2018
Available online 19 November 2018
0306-4565/

recognition and/or directed to inactivate the host effector mechanisms (Brivio et al., 2004, 2006; Mastore and Brivio, 2008; Eleftherianos et al., 2010).

Bt is a Gram-positive, spore-forming bacterium which, after ingestion by the insect, releases δ-endotoxins. These toxins, interacting with epithelial cells, drastically affect the absorption of insect midgut (Gill et al., 1992). The damage to the midgut epithelium allows the passage of the bacterium to the haemocoel cavity, exposing the pathogen to the host immune system (Jung and Kim, 2007; Dubovskiy et al., 2008). When it reaches the haemocoel cavity, by means of its secondary metabolites, it can immune depress the host. Thanks to their high lethality, *Bt* sub-species and their toxins are widely used to control insect pests (Lacey et al., 2015). Although commercial *Bt* var. *kurstaki* is generally targeted against susceptible Lepidoptera pests, recent works demonstrated its efficacy against Diptera (Lonc et al., 2001; Lysyk and Selinger, 2012; Cossentine et al., 2016).

Among various environmental factors, temperature is certainly one of those most affecting the efficacy of both EPNs and *Bt*. Temperature variations can directly influence the physiology of nematodes and, consequently, the outcome of their life cycle. In particular, low temperatures can cause the reduction of metabolic levels impairing motility and rate of host penetration (Hazir et al., 2001; Bornstein-Forst et al., 2005; Gingold et al., 2013; Ali and Wharton, 2015). Despite literature data being sometimes contradictory, the lethality of *Bt* is affected by temperature variations and various hosts show a different susceptibility to the bacteria at different temperatures. *Bt* reduces its effects against the Diptera *Stomoxys calcitrans* as the temperature increases (Lysyk and Selinger, 2012). The Lepidoptera *Choristoneura rosaceana* is more vulnerable to the bacteria at 25 °C with respect to lower temperatures (Li et al., 1995); conversely, the pine processionary *Thaumetopoea wilkinsoni* (Lepidoptera) showed the highest mortality when *Bt* was administered at 15 °C (Yilmaz et al., 2013). The lethal effects of the bacteria occurred sooner and progressed rapidly with increasing temperature in *Lymantria dispar* (Lepidoptera), although the highest level of mortality decreased with increasing rearing temperature (van Frankenhuyzen et al., 2008).

After parasite or bacteria intrusion, insects can trigger defensive responses as nodulation and melanotic encapsulation. Specifically, the latter represents the fastest and most effective process to neutralise and eliminate intruders, and both these mechanisms depend on the activation of the proPO system cascade that, in turn, cleaves the prophenoloxidase into its active form (Marmaras et al., 1996; Cerenius et al., 2008). Moreover, the presence of bacteria such as *Bt* or EPNs symbionts in the haemolymph can be also counteracted by the action of specific enzymes such as lysozymes, i.e., a constitutive factor that quantitatively increases in the haemolymph because of an infection (Yu et al., 2002; Andrejko et al., 2008).

Changes of environmental temperature can also affect the immune responses of insect hosts (Adamo and Lovett, 2011; Wojda and Taszłow, 2013; Wojda, 2017). In some cases, warmer temperatures increase insect immune defences by means of the increase in activity of both phenoloxidase (i.e., PO activity) and lysozyme, other than trigger cell-mediated immunity (Catalán et al., 2012; Murdock et al., 2012). As observed in *Drosophila*, cold exposure also modulates basal immunity processes, increasing antibacterial peptides and decreasing PO activity, though resistance to pathogens and parasites seem to not be affected (Salehipour-Shirazi et al., 2017).

Insect resistance involves broad aspects of host physiology and conditions, which are shaped by both genetic and environmental variations that often interact in nonlinear ways (Schulenburg et al., 2009). From works on various host-parasite systems, minor changes in temperature can have striking effects on the outcome of their interactions; indeed, the environmental temperature can deeply affect overall resistance to a wide diversity of parasites such as nematodes (Menti et al., 2000) and bacteria (Lazzaro et al., 2008).

A correct use of bio-insecticides like EPNs and *Bt* in areas with different environmental temperature ranges and changing climatic conditions should consider the possible differences in their efficacy and the possible change of insects' physiology due to temperature variations.

In order to acquire useful information on host-parasite relationships in the context of climate change, we aimed to obtain further data on the performance of EPNs and *Bt* and on basal immunity of two insect models when reared at different temperatures (i.e., 10, 20 and 30 °C). We first compared the action of three EPNs (i.e., *S. feltiae*, *S. carpocapsae*, and *H. bacteriophora*) and *Bt* against *Galleria mellonella* (Lepidoptera) and *Sarcophaga africa* (Diptera); two insect models known as honeybee pest and vector of human and animal diseases respectively. In addition, we studied the modulation of the basal humoral immunity of the insects' host at the different temperatures investigated. Specifically, considering the central role of the insect proPO system in the host immune responses and the activity of lysozyme in bacterial clearance, we evaluated the influence of the temperature on the PO and lysozyme activity in the host haemolymph. We think that this work may provide knowledge about the influence of temperature on the host susceptibility to different entomopathogens and on the host immune system, and that, in turn, this knowledge may be used to improve biological control techniques.

2. Materials and methods

2.1. Reagents and instruments

Reagents and instruments were purchased from Bio Rad Laboratories (Detroit, MI, USA), Sigma Chemicals (St. Louis, MO, USA), Merck Millipore Ltd (Tullagreen, Cork, Ireland). All buffers and solutions were heat sterilised or filtered on 0.22 μm syringe filters.

2.2. Bio-insecticides and target insect models

For propagation assays we used commercially available EPNs and *Bt*: *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* were supplied by Bioplanet srl (Cesena, Italy) as Nemopak® (SF, SC, and HB, respectively); *Bt* sub-species *kurstaki* was purchased from Chemia SpA (Sant'Agostino, Italy) as a product commercially named Rapax®.

EPNs, provided at the third infective juvenile stage, dehydrated, and mixed with inert material, were stored at 4 °C to keep parasites in cryptobiosis. *Bt* was supplied as a suspension: 100 gr of product contain 18.8 gr of bacteria. For all the assays *G. mellonella* and *S. africa* were used as insect models as previously mentioned. Both the species were fed with sugar and water ad libitum and kept under controlled conditions: 26 ± 1 °C temperature, 70 ± 10% relative humidity, and 12:12 h of photoperiod. All the assays were carried out with healthy individuals at comparable growth stages (4th–5th instar).

2.3. Propagation assays

As natural infestation method, we implemented a white trap tool to evaluate the lethality of the three EPNs (i.e., *S. feltiae*, *S. carpocapsae* and *H. bacteriophora*) and *Bt* against *G. mellonella* and *S. africa*. Briefly, two grams of nematodes were diluted in 100 mL of dechlorinated water, or 150 μL of *Bt* were diluted in 50 mL of dechlorinated water. The EPNs solution (6 mL) was dispensed on a filter paper placed in a 9 cm diameter Petri dish (i.e., white trap), otherwise the *Bt* solution (6 mL) was spread on food (honey plus cereal for *G. mellonella* or meat for *S. africa*) and placed in the white trap; then, ten larvae were transferred in each white trap. All the insects were kept under controlled conditions (70 ± 10% relative humidity, 12:12 h of photoperiod) in thermostatic incubators (Memmert GmbH + Co. KG, Schwabach, D) at different temperatures (i.e., 10, 20, and 30 °C) and the mortality rate was evaluated at intervals of 12 h, for 60 h. For each bio-insecticide and temperature investigated, three replicates were carried out. As controls, in

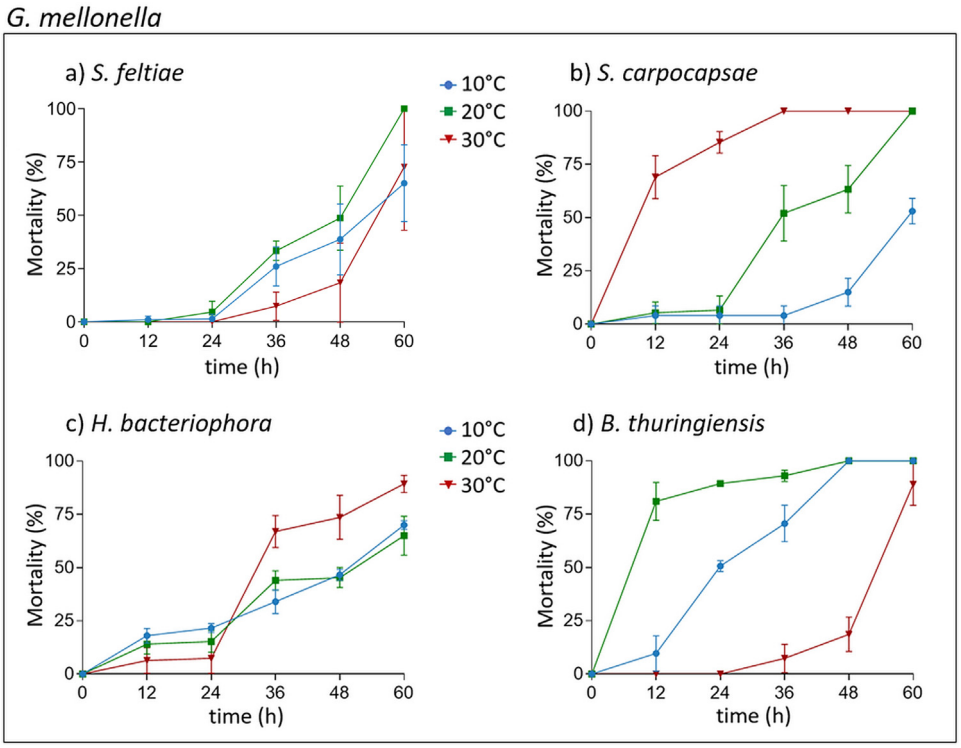


Fig. 1. Mortality rate of *G. mellonella* after exposure to *S. feltiae* (a), *S. carpocapsae* (b), *H. bacteriophora* (c), and *Bt* (d) at different temperatures (10, 20, 30 °C) for 60 h. Larvae in control assays showed a 0% mortality.

all the assays dechlorinated water without bio-insecticides was used.

2.4. Haemolymph collection

Haemolymph, 30 μL/larvae, was bleeded from punctured prolegs of last instar larvae. To avoid the undesired activation of prophenoloxidase, samples analysed for PO activity were flushed out in ice-cold Eppendorf tubes, while samples analysed for lysozyme activity were supplemented with a few crystals of phenylthiourea. Whole haemolymph samples were processed by two low-speed centrifugations (200*g* for 3 min) to remove cells and tissue debris by a refrigerated Eppendorf 5804 R (Eppendorf, Hamburg, Germany). The supernatants cell-free fractions (CFF) were immediately assayed for enzymatic activity.

2.5. PO activity

The PO activity in the haemolymph from untreated *G. mellonella* and *S. africa* larvae incubated at different temperatures (10, 20 and 30 °C) for 24 h was evaluated by spectrophotometric analysis of CFF samples, using L-Dopa as substrate. Time courses of PO activity in the haemolymph from *G. mellonella* and *S. africa* were recorded as formation of dopachrome from L-Dopa substrate, to determine the relative activity of the enzyme. All the assays were carried out with 10 μL of CFF in 1 mL of L-Dopa buffer (8 mM L-Dopa in 10 mM Tris–HCl, pH 7.2). 200 μL of sample/well were read for absorbance (Abs) in a flat-bottomed 96 well-plates by Bio Rad iMark™ Microplate Absorbance reader. Changes in Abs were recorded at 450 nm, at 5 min intervals for 45 min, at 20 °C. The L-Dopa buffer was used as blank, and in all samples PO relative activity was defined as $\Delta Abs_{450} \cdot 45\ min^{-1} \cdot 10\ \mu L^{-1}$. For each temperature three replicates were carried out; each replicate was composed of the haemolymph from three larvae. For each replicate three Abs readings were performed and then the average value was considered.

2.6. Lysozyme activity

An enzymatic assay was carried out to measure the rate of lysozyme activity (Units min^{-1} mL^{-1}) in the haemolymph samples from untreated *G. mellonella* and *S. africa* larvae incubated at different temperatures (10, 20 and 30 °C) for 24 h. One unit of lysozyme activity is defined as the change of 0.001 units of Abs at 450 nm per minute of a suspension of the Gram-positive bacteria *Micrococcus luteus* (ΔAbs_{450}). The lysozyme present in the haemolymph damages the bacterial cells wall, decreasing the turbidity of the sample and therefore its Abs. Briefly, a suspension of *M. luteus* (0.45 mg mL^{-1} in 30 mM phosphate buffer, pH 7.2) with 0.6–0.7 of optical density was used as substrate, then 25 μL of CFF were added to the bacteria suspension. The Abs of samples was read at 450 nm and recorded at 30 s intervals for 2.5 min, in a flat-bottomed 96 well-plates by a Bio Rad iMark™ Microplate Absorbance reader. For each temperature three replicates were carried out; each replicate was constituted of the haemolymph from three larvae. For each replicate four Abs readings were performed and then the average value was considered. *M. luteus* without haemolymph addition was used as control.

2.7. Statistical analysis

For each bio-insecticide (*S. feltiae*, *S. carpocapsae*, *H. bacteriophora*, and *Bt*) and temperature (10, 20, and 30 °C) through the time interval analysed (12, 24, 36, 48 and 60 h), we calculated the insects' mortality as average ± standard deviation of the three replicates. For each bio-insecticide-target insect pair, we applied the two-way analysis of variance (ANOVA) considering temperature and time as variables and the percentages of insect mortality as observations. Before the ANOVA the percentage data were *arcsin*-transformed. After the ANOVA we used the Tukey HSD *post-hoc* test to detect possible significant ($p < 0.05$) differences of insect mortality at different temperatures and times. Moreover, we applied the one-way ANOVA followed by the Tukey HSD *post-hoc* test to detect possible significant ($p < 0.05$) differences of the

PO and lysozyme activity at the three temperatures investigated. All the statistical analyses were performed using XLSTAT2011 software.

3. Results

3.1. Effects of temperature on the performance of entomopathogens

In all cases, statistically significant differences of the larvae mortality were recorded at different temperatures and durations of exposure to the four bio-insecticides, and for the interaction between these two variables (two-way ANOVA, all $p < 0.05$, see Tables SM1 and SM2 in the Supplementary material), except for the assays with *S. feltiae* against *G. mellonella* and *S. africa*. In these two cases, significant differences were only related to the period of treatment and, for the former, also to temperature (Tables SM1 and SM2).

The susceptibility of *G. mellonella* to *S. feltiae* is evident, mainly at 20 °C (Fig. 1a). At this temperature, after 48 h of treatment, the mortality was 48% on average, and after 60 h it reached 100% (Fig. 1a). However, a significant difference was only recorded at 60 h between 10 and 20 °C (Tukey test, $p = 0.015$, Table SM3). The Diptera *S. africa* was less affected by *S. feltiae*; the mortality rate never exceeded 50% on average and this value was reached at 30 °C after a prolonged exposure time (i.e., 60 h) (Fig. 2a).

Conversely, the other EPN species belonging to the *Steinernema* genus (i.e., *S. carpocapsae*) was significantly more lethal at the highest temperature tested for both the targets (Figs. 1b and 2b) (Tukey test, $p < 0.05$ in most cases, see Tables SM3 and SM4). After 36 h of exposure at 30 °C, *G. mellonella* showed a mortality of 100% (Fig. 1b). In the case of *S. africa* (Fig. 2b) an average mortality of approximately 75% was detected at 60 h.

The third EPN investigated (Figs. 1c and 2c), *H. bacteriophora*, had a behaviour similar to that of *S. carpocapsae* as the average larvae mortality resulted higher at the highest temperature tested. At 30 °C after 60 h of exposure, the mortality of both insects (Figs. 1c and 2c) was over 80% on average and significantly different from that detected at lower temperatures (Tukey test, all $p < 0.05$ except for 30 vs 10 °C in case of *G. mellonella*, see Tables SM3 and SM4).

The results of the *Bt* assays (Figs. 1d and 2d) show a significantly higher efficacy of the bacteria at 10 and 20 °C for *G. mellonella* and at 30 °C for *S. africa* (Tukey test, $p < 0.05$ in most cases, see Tables SM3 and SM4). At 20 °C, the mortality of *G. mellonella* reached approximately 80% in only 12 h (Fig. 1d). *Bt* was ineffective against *S. africa* at both 10 and 20 °C even though, after 48 h of exposure at 30 °C, it caused the mortality of more than 65% of the Diptera larvae (Fig. 2d).

3.2. Effects of temperature on the PO activity

As shown in Fig. 3a, the PO relative activity in the cell-free haemolymph samples of *G. mellonella* significantly differed between all the three temperatures investigated (one-way ANOVA, $F = 95.075$, $p < 0.0001$, Table SM5). In particular, the PO activity was significantly higher at 30 °C (Tukey test, 30 vs 20 °C: $p < 0.0001$; 30 vs 10 °C: $p = 0.001$, Table SM5), though it was also significantly higher at 10 °C than at 20 °C (Tukey test, $p = 0.001$, Table SM5). In general, *S. africa* (Fig. 3b) was less influenced by the conditioning at different temperatures (one-way ANOVA, $F = 16.736$, $p = 0.004$, Table SM5). However, a significantly higher activity was also recorded at 30 °C in this case (Tukey test, 30 vs 20 °C: $p = 0.007$; 30 vs 10 °C: $p = 0.005$, Table SM5); conversely the difference between the activity at 10 and 20 °C did not yield significant results (Tukey test, $p = 0.904$, Table SM5).

3.3. Effects of temperature on the lysozyme activity

In all the lysozyme assays with *G. mellonella* and *S. africa*, an Abs decrease corresponds to a higher lysozyme activity (Fig. 4). As shown in Fig. 4a, the relative activity of the haemolymph lysozyme of *G. mellonella* significantly increases with the temperature (one-way ANOVA, $F = 51.965$, $p < 0.0001$, Table SM6). A negligible activity was recorded at 10 °C ($3.44 \cdot 10^2$ Units mL^{-1} min^{-1}) since no significant difference was

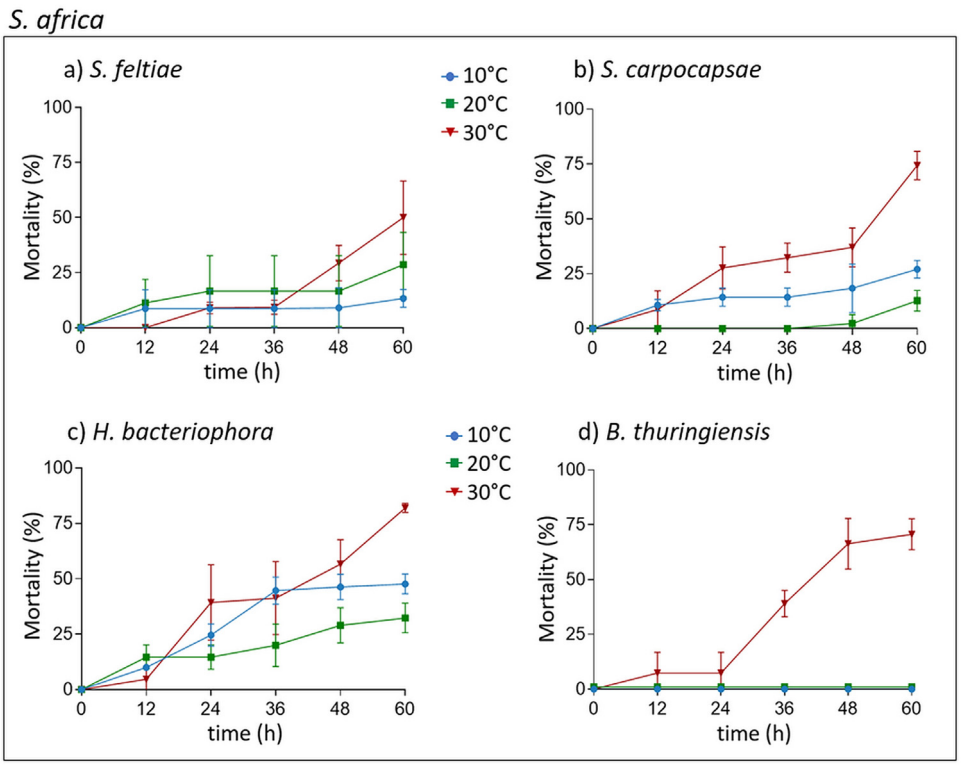


Fig. 2. Mortality rate of *S. africa* after exposure to *S. feltiae* (a), *S. carpocapsae* (b), *H. bacteriophora* (c), and *Bt* (d) at different temperatures (10, 20, 30 °C) for 60 h. Larvae in control assays showed a 0% mortality.

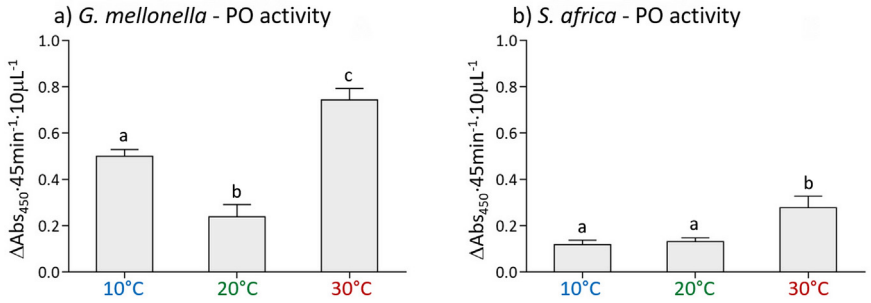


Fig. 3. PO relative activity in haemolymph of *G. mellonella* (a) and *S. africa* (b) after conditioning at various temperatures (10, 20, 30 °C). Different letters above the bars (a, b, and c) indicate a statistically significant ($p < 0.05$) difference between samples.

found with respect to the control (i.e., Tukey test, *M. luteus* vs 10 °C: $p = 0.675$, Table SM6), while significantly higher activities (Tukey test, all $p \leq 0.005$, see Table SM6) were detected at 20 °C ($1.27{\cdot}10^3$ Units mL^{-1} min^{-1}) and overall at 30 °C ($2.28{\cdot}10^3$ Units mL^{-1} min^{-1}). Unlike *G. mellonella*, *S. africa* (Fig. 4b) possesses a negligible lytic activity at any temperature tested (i.e., $1.62{\cdot}10^2$; $1.04{\cdot}10^2$ and $1.68{\cdot}10^2$ Units mL^{-1} min^{-1}, for 30, 20, and 10 °C treatments, respectively) and no significant differences were recorded between all the samples (one-way ANOVA, $F = 2.202$, $p = 0.165$, Table SM6).

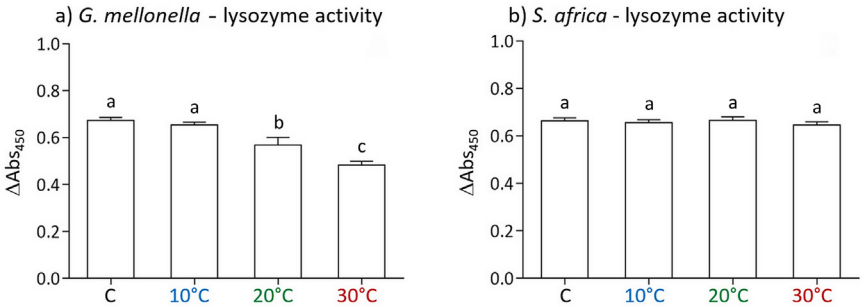
4. Discussion

As we mentioned in the introduction, the current climate change is leading to the expansion and modification of the habitat for many insect pests (Bebber, 2015) and to shifts in host-parasite interactions (Rohr and Palmer, 2013). Most host-parasite interactions are indeed mediated by physiological (mainly immunological) processes which, in turn, can be influenced by environmental temperature. From the current literature, it is clear that establishing common models of response to changes in environmental temperature is difficult, since there are discordant data even within ectotherm species. One of the reasons for this inhomogeneity is related to the maintenance conditions in laboratories in which physical parameters cannot exactly reflect those of the habitat of the species, other than the different experimental procedures performed (Terblanche et al., 2007; Santos et al., 2011; Gallego et al., 2016).

In this work, we first analysed the performance at different temperatures of four entomopathogens (*S. feltiae*, *S. carpocapsae*, *H. bacteriophora,* and *Bt*), used as bio-insecticides, against the Lepidoptera *G. mellonella* and the Diptera *S. africa*, belonging to orders comprising many phytophagous and vectors species. Since it is well known that temperature modulates biological processes of both hosts and parasites and that they function optimally within certain temperature ranges (Kung et al., 1991; Mahar et al., 2005; Shapiro-Ilan et al., 2006; Lacey, 2007; Lee et al., 2016), we expected a different infectivity level of the entomopathogens at the three different temperatures investigated (10, 20 and 30 °C).

Our assays showed a higher susceptibility of *G. mellonella* to all entomopathogens with respect to *S. africa*, reaching higher average mortality values within 60 h of treatment. *S. carpocapsae* already showed good infectivity (i.e., > 50% mortality) at high temperature (30 °C) at 12 h of treatment, likely due to a higher activity of the parasite and a better growth condition of its symbionts, *Xenorhabdus nematophila* (Chen et al., 1996; Wang et al., 2008). The infectivity of *S. carpocapsae* significantly decreased at lower temperatures (20 and 10 °C). Grewal et al. (1994) observed that this nematode is able to penetrate insects also at temperatures up to 10 °C but remains inactive within its hosts for long periods and resumes its life cycle only at warmer temperatures. Despite belonging to the same genus, *S. feltiae* had a good level of infectivity against *G. mellonella* only at 60 h of treatment at all the investigated temperatures, and caused the 100% of host mortality only at 20 °C. This result is partially in agreement with the literature which suggests that this nematode is more lethal at temperatures near to 20 °C (Chen et al., 2003; Mahar et al., 2005; Laznik et al., 2009). *S. feltiae* is indeed a temperate species that has evolved as an active nematode at cool temperatures (Hominick and Briscoe, 1990; Wright, 1992). However, some authors (Wright, 1992; Grewal et al., 1994) suggest that, thanks to its features, *S. feltiae* could also affect insects that are more active during winter. The Canterbury isolated strain, for instance, is infective at low temperatures, being able to kill *Wiseana cervinata* (Lepidoptera) at 10 °C (Wright and Jackson, 1992). Unlike *S. feltiae*, *H. bacteriophora* has a tropical or sub-tropical origin, thus its thermal preferences are specific to warmer climates (Flanders et al., 1996; Stuart et al., 2015). Accordingly, we observed that *H. bacteriophora* had significantly higher activity against *G. mellonella* at 30 °C, starting from 36 h of treatment.

EPNs are also reported to be lethal against Diptera (Jagadale et al., 2004; Jess et al., 2005; Georgis et al., 2015). Several works have showed the adverse effects of *Steinernema* spp. against the Diptera



lysozyme relative activity (Units · mL^{-1})

T= 10°C	T= 20°C	T= 30°C
$3.44{\cdot}10^2$	$1.27{\cdot}10^3$	$2.28{\cdot}10^3$

lysozyme relative activity (Units · mL^{-1})

T= 10°C	T= 20°C	T= 30°C
$1.68{\cdot}10^2$	$1.04{\cdot}10^2$	$1.62{\cdot}10^2$

Fig. 4. Lysozyme activity in haemolymph of *G. mellonella* (a) and *S. africa* (b) after conditioning at various temperatures (10, 20, 30 °C). The lytic activity of lysozyme was recorded as decrease in turbidity of a *M. luteus* suspension (control, C). Different letters above the bars (a, b, and c) indicate a statistically significant ($p < 0.05$) difference between samples. Tables below the graphs show the lysozyme relative activities as Units mL^{-1}.

Bactrocera oleae, Ceratitis capitata and *Rhagoletis indifferens* (Lindegren et al., 1990; Gazit et al., 2000; Yee and Lacey, 2003). However, in the literature, there are no studies on the pathogenicity of EPNs on *S. africa*. According to our results, when *S. carpocapsae* or *H. bacteriophora* were administered at 30 °C, both revealed a good level of infectivity on *S. africa*, even if they required a treatment of 60 h. As confirmed by the literature (Rohde et al., 2010; Pervez et al., 2015), *S. carpocapsae* and *H. bacteriophora* display the highest pathogenicity between 25 and 30 °C. Specifically, *S. carpocapsae* is highly lethal within this temperature range against the larvae of the Diptera *C. capitata*, *Dacus dorsalis,* and *Dacus cucurbitae* (Lindegren and Vail, 1986; Patterson Stark and Lacey, 1999), and *H. bacteriophora* against the Diptera *Lucilia cuprina* (Molyneux, 1984). Unlike these two EPNs, *S. feltiae* showed a low level of infectivity (i.e., ≤ 50% mortality) at all the temperatures tested within 60 h of treatment. The average mortality values were lower at 10 °C, although not significantly different from those detected at 20 and 30 °C. In other species of Diptera, such as *D. melanogaster*, *S. feltiae* was highly infective at 25 °C, showing a low lethal effect at lower temperatures, while it was able to kill *Delia radicum* larvae at 10 °C (Patterson Stark and Lacey, 1999; Chen et al., 2003; Linder et al., 2008).

In general, other than changes in EPNs infectivity, variations in temperature can cause changes in host-seeking behaviour and in the time necessary to infect the host (Molyneux, 1984). Also, the attractive stimuli produced by the insect can vary with temperature, affecting the chemotaxis processes (Pye and Burman, 1981; Kaya, 1993). Moreover, the different levels of infectivity described for *Steinernema* spp. and *Heterorhabditis* spp. at various temperatures can be due to the physiology of their symbionts, as bacteria health and growth are affected by temperature variations (Dunphy and Webster, 1988).

As for *Bt*, its infectivity at different temperatures had an opposite trend in the two target insects. The bacterium had a significantly higher lethality on *G. mellonella* at 20 and 10 °C than at 30 °C, while when it was administered to *S. africa*, significantly higher mortality was observed at just 30 °C. This higher lethality at 30 °C can be expected, since the optimal growth of *Bt*, the solubility and absorption of its crystal toxins, and the feeding rate increase with the temperature (Wraight et al., 1981). The influence of temperature on the bacterial growth has also been suggested by van Frankenhuyzen (1994), who evaluated the pathogenesis of *Bt* on *Choristoneura fumiferana* (Lepidoptera).

The main results of the assays carried out on *G. mellonella* and *S. africa* with the four entomopathogens at various temperatures and within 48 h of treatments are summarized in Table 1. In the context of climate change, these results show that entomopathogens like *S. carpocapsae* and *H. bacteriophora* could be favoured by the predicted increase in the global surface temperature (Björkman and Niemelä, 2015), while the survival of hosts such as *S. africa* could be affected by a wider range of parasites. Our results may also constitute basic guidelines useful to select the adequate bio-insecticide under varying climatic conditions for field applications.

Concomitantly with the study of entomopathogens' lethality, we have examined the effects of temperature on the basal immunity of the target insects to investigate the possible association with an altered activity of the host immune system. Although the immune defences are energetically costly, the insects' immune system can be modulated to operate at different temperatures to counteract parasites or bacteria with different thermal performances (Moret and Schmid-Hempel, 2000; Adamo, 2004; Xu and James, 2012). Thus, our assays have been carried out to evaluate how thermal conditioning modulates the basal PO and lysozyme activity, both present in the humoral fraction of the insects' haemolymph and known as the fastest mechanisms and most effective defence processes against metazoan parasites and microorganisms (Hultmark, 1996; Gao and Fallon, 2000; Brivio et al., 2002; Lemaitre and Hoffmann, 2007; Cerenius et al., 2008).

Table 1
Cumulative table of the efficacy (referred to 48 h post administration) of *S. feltiae* (Sf), *S. carpocapsae* (Sc), *H. bacteriophora* (Hb) and *B. thuringensis* (Bt) on *G. mellonella* (Gm) and *S. africa* (Sa), after conditioning at 10, 20 and 30 °C.

	10 °C				20 °C				30 °C			
	Sf	*Sc*	*Hb*	*Bt*	*Sf*	*Sc*	*Hb*	*Bt*	*Sf*	*Sc*	*Hb*	*Bt*
Gm	+	–	+	+ + +	+	+ +	+	+ + +	–	+ + +	+ +	–
Sa	–	–	+	–	–	–	+	–	+	+	+ +	+ +

–: Mortality < 25%; +: 25 ≤ Mortality ≤ 50%; + +: 50 ≤ Mortality ≤ 75%; + + + : Mortality > 75%.

Our data show the influence of temperature mainly on the *G. mellonella* PO and lysozyme activity. The PO activity increased moderately at 10 °C and strongly at 30 °C, whereas at 20 °C it kept close to the physiological activity (Zdbicka-Barabas and Cytryńska, 2010). In *S. africa,* the PO activity significantly increased only at 30 °C. Moreover, in *G. mellonella* haemolymph, we recorded a significant increase of the lytic properties from 10 to 30 °C, while in *S. africa* we did not observe significant variations of the lysozyme activity at any temperature assayed. As we found in *G. mellonella*, an influence of the temperature on the physiological PO and lysozyme activity in the haemolymph was also observed in the Orthoptera *Gryllus texensis* (Adamo and Lovett, 2011), the Coleoptera *Tenebrio molitor* (Catalán et al., 2012), the Diptera *Anopheles stephensi* (Murdock et al., 2012), the Lepidoptera *Ephestia kuehniella* (Mostafa et al., 2005), and other species.

Unlike from studies that have examined individual components or processes of the complex system entomopathogen-host (Laughton et al., 2017), in our work we used a wider approach to investigate the effects of temperature on both hosts and entomopathogens, also considering their synergistic relationships. Unlike *Bt*, which is a single micro-organism, an in-depth understanding of the EPNs' relationships with their hosts is complicated by the presence and the role played by their symbionts bacteria, which combine with the nematode their lethal effects in a tripartite interplay (Sicard et al., 2004; Hallem et al., 2007; Toubarro et al., 2013; Mastore et al., 2015; Brivio et al., 2018). Along with the changes of temperature, the complex interactions among all these species and the various processes involved can cause different immune responses, sometimes leading to divergent results (Björkman and Niemelä, 2015). Even when in the presence of an intensified immune response of the host, as we detected in *G. mellonella* at 30 °C, the evasive and depressive strategies of EPNs such as *S. carpocapsae* and *H. bacteriophora* could be responsible for a successful parasitisation. Considering the increase of PO activity at higher temperatures, we expected a better efficiency of the hosts to encapsulate parasites (Dubovskiy et al., 2016); conversely, the observed increase did not produce the expected effects. The elusive strategies of the parasites, in particular their ability to evade non-self recognition by means of the peculiar properties of their cuticle (Peters et al., 1997; Castillo et al., 2011; Cooper and Eleftherianos, 2016; Brivio and Mastore, 2018), probably defused the potential of the increased immune activity of the host. Otherwise, the delayed effects of *Bt* against *G. mellonella* and the high mortality observed in *S. africa* at 30 °C could be associated to the different lysozyme activity observed in the two insects at this temperature. The survival of *G. mellonella* could be due to the action of the enzyme on the wall of this Gram positive bacterium (Hultmark, 1996; Callewaert and Michiels, 2010) leading to a reduced bacterial proliferation in the host haemolymph.

5. Conclusions

Our data suggest that temperature changes can alter the relationships between entomopathogens and their hosts, thus affecting the efficacy of the bio-insecticides on the target insects in specific ways in different species. Temperature can also influence the host basal immune response even if, in some instances, the enhancement of immune activity does not seem to be the main factor responsible for an increased

resistance to entomopathogens. Therefore, the balance between the success of a bio-insecticide and the insect survival can differ as a result of environmental variations, which could be responsible for physiological changes of both the host and the entomopathogen.

Although we are aware that further studies are required to deepen the complex relationships between entomopathogens and their hosts in a context of climate change, we believe that our work may provide useful evidence from both a theoretical and applied viewpoint.

Acknowledgments

We are grateful to Michela Curradi Ph.D. for reading the manuscript and advices.

Funding

This research was funded by FAR funds from University of Insubria, Italy.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jtherbio.2018.11.006.

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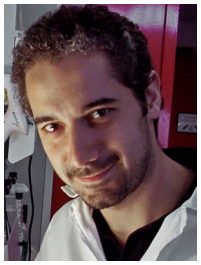

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CHAPTER 5

Work in progress

5.1 An emerging pest

5.1.1 The "spotted wing fly" Drosophila suzukii

The spread of insect pests has represented, and will surely represent in the future, an ever-increasing problem that will require a great attention and the use of innovative resources to keep it under control. In recent years, among the insect species considered to be very dangerous for crops, the "spotted wing fly" *Drosophila suzukii* has become widespread in Europe, this insect is considered one of the major invasive pests of small fruits (Asplen et al, 2015). Many soft-skinned fruits have been identified in Europe and Asia to be susceptible targets of this Diptera (Arnó et al, 2016; Kenis et al, 2016); blueberries, raspberries, strawberries, grapes and peaches are attacked by this insect (Walsh et al. 2011). *Drosophila suzukii* females lay the eggs inside the fresh fruits using their serrated ovipositor that help them to penetrate the fruit soft skin (Hamby et al, 2016), for this reason, they have a wide food niche compared to other flies, indeed they can infest healthy ripening fruits in addition to the fallen ones.

As mentioned in literature, the first control measure is by cultural and chemical methods (Asplen et al, 2015), these products are not compatible with integrated pest management (IPM) techniques (Van Timmeren and Isaacs 2013) due to the effects on the environment, so alternative control methods are needed (Wiman et al, 2016). Nowadays, the studies for the biological control of *D. suzukii* are focused on natural enemies, including predators (Gabarra et al, 2015), parasitoids (Chabert et al, 2012) and entomopathogenic agents (fungi and nematodes). Entomopathogenic nematodes (EPNs) can infect a wide range of pests under laboratory conditions and can cause high mortality. Even if many studies on *Drosophila melanogaster* reported levels of 80% of infection with *S. carpocapsae*, *S. feltiae* or *H. bacteriophora* (Dobes et al, 2012), only few studies about the efficacy of EPNs against the spotted wing fly *D. suzukii* are available.

5.1.2 Summary of the preliminary assays

In order to acquire knowledge about the immune response of *D. suzukii*, we have planned some preliminary experiments to test the efficacy of bio-insecticides and the immuno response of the host.

To evaluate the basal phagocytotic activity of *Drosophila* hemocytes *S. aureus* conjugated with the fluorescent pHrodo probe has been used (with the method used in the published attached paper what paper, specify) and to assay the elusive properties of the entomopathogenic bacteria, we incubated host cells with GFP-labeled *X. nematophila* (with the green fluorescent protein genetically expressed).

Moreover, to assay the immunoevasive/depressive properties of S. carpocapsae, we have carried out encapsulation assays with the entomopathogen nematode, live or dead and, to investigate the mimetic role of the body surface, with lipase-treated nematodes. Finally, to analyze the activity of the proPO system, we infected the larvae with *X. nematophila* and a with a suspension of Gram-positive/Gram-negative bacteria.

5.1.3 Results

Regarding the phagocytosis process, in *in vitro* experiment we have observed the normal activity of the host hemocytes that are able to engulf *S. aureus* cells. The pHrodo probe allows to evaluate correctly cells directly involved in the phagocytosis process (fig. 20).

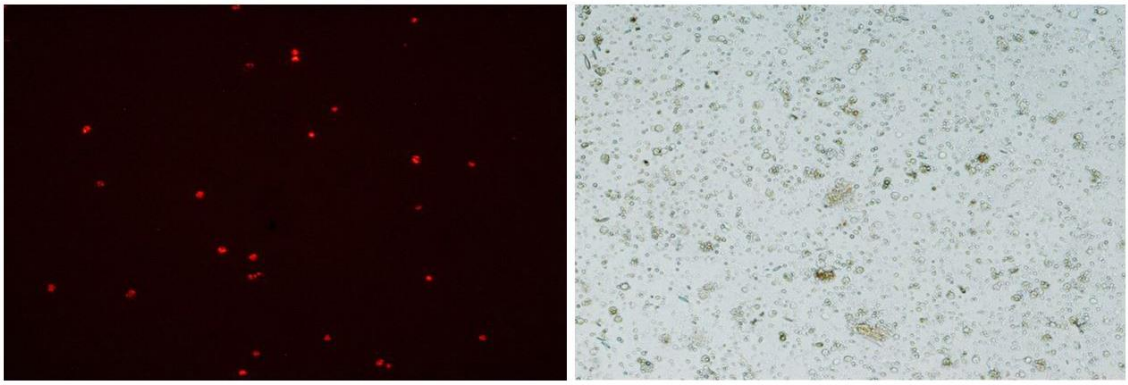


Fig. 20 - Phagocytosis activity: S. aureus conjugated with pHrodo probe. In red bacteria inside the host cells.

In *in vitro* experiments, performed with *X. nematophila* (with GFP luminescent probe), we observed the bacteria outside the hemocytes, the entomopathogen bacteria seems not to be detected by the host cells (fig. 21) as observable by the elongated shape and by the movement of the bacteria cells.

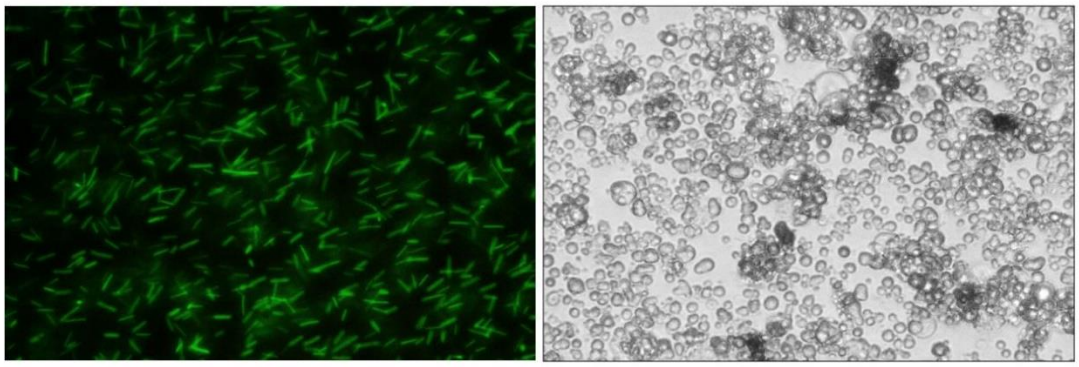
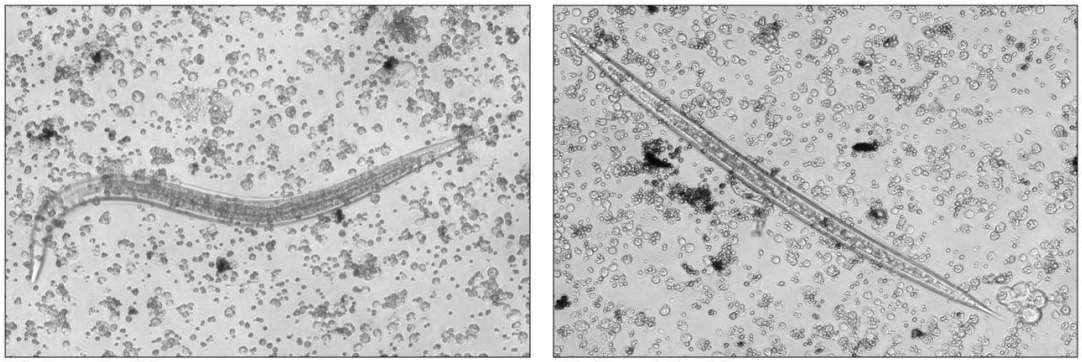


Fig. 21 - Phagocytosis activity: GFP-labeled X. nematophila. The bacteria (green) are observable outside the host cells.

Considering that, normally, in other insect species, *S. carpocapsae* is not detected by the hemocytes, we verify if the nematode was able to evade the recognition by the hemocytes of Drosophila. As shown in the fig. 22 (left), after 2 hrs of incubation live nematodes were not encapsulated by the cells. To verify if the lack of recognition could be due to secretion activity of the worm, we repeated the assays with dead *Steinernema* and also in this case the cells were not able to recognize and encapsulate the target.



2h live Sc 10x 2h Dead Sc 10x

Fig. 22 - Encapsulation process: the EPN S. carpocapsae is not detected by the host hemocytes.

To assess if the body surface (cuticle and epicuticle) played a role in the elusive process, we pre-treated the nematodes with a specific enzyme to modify the body-surface of the worm; lipase treatment removed the lipidic moiety of the structure. Fig. 23 shows the effects of the treatment; in the micrograph are clearly visible the early layers of hemocytes attached to the nematode surface that start forming a capsule.

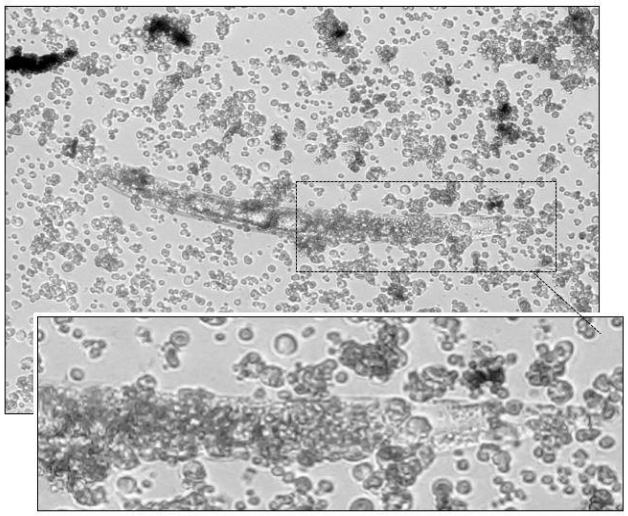
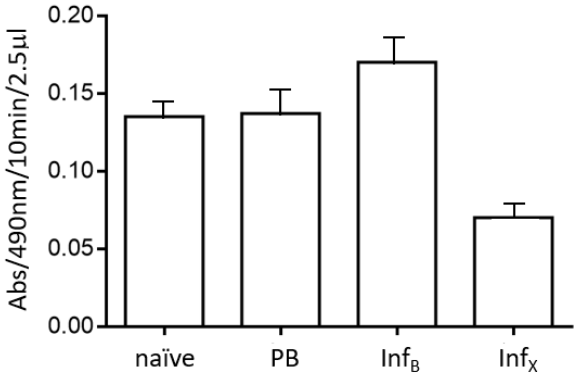


Fig. 23 - Encapsulation process: EPN treated with lipase shows host hemocytes attached to its surface.

Finally, we evaluated the proPO system activity of *Drosophila*, in this assay we used hemolymph extracted from untreated larvae (naïve), larvae treated with phosphate buffer (PB), larvae infected with a suspension of Gram-positive/Gram-negative bacteria (Inf_B) and larvae infected with the EPN's symbionts, *X. nematophila* (Inf_X). The results in fig. 24 show the increase of activity after the infection with non-pathogenic bacteria (Inf_B), and the inhibitory effects induced by the presence of *X. nematophila* (Inf_X); the latter result confirmed an interference of the symbionts with the immuno response of the host.



sample	Abs
naive	0.135
PB	0.137
inf_B	0.170
inf_X	0.070

Fig 24 - proPO activity in hemolymph of Drosophila suzukii after various treatments. Naïve: untreated larvae; PB: phosphate buffer injected; InfB: infected with bacteria mixture; InfX: infected with X. nematophila.

After these preliminary assays aimed to evaluate the immune response of *D. suzukii, a* further investigation will be carried out to evaluate synthesis and activity of antimicrobial peptides (AMPs), since these molecules play a central role in the bacteria clearance. This assay can be further supported by analysis of AMPs genes expression, to quantify both quality and quantity of the production after different stimuli.

5.2 Conclusions

Insects are becoming increasingly important in many areas of scientific research, from biological alternatives to pesticides to resources against the growing hunger in the world, from antibiotics research to use as valid alternative biological models. Investigating the effects induced by nematode-bacterial complexes, represents a valid starting point to improve and develop innovative methods for field treatments, which may in the future provide for the direct administration of compounds isolated from bioinsecticides, instead of organisms in toto. Studies at molecular and cellular level, in the field of biological control, allow us to deeply understand the interactions between the target organism and its competitor, and this will allow us to improve treatments making them more effective, less expensive and simple to use. The improvements could increase the dissemination of these eco-sustainable techniques to an increasing number of users. The advisable goal is the replacement, or at least a reduction, of chemical pesticides with alternatives more conservative for the environment, animals and human health. The problem of the spread of pest species will undoubtedly lead to increased interest from the scientific world which will certainly bring further future developments in the field of biological control.

Despite the progress of effective techniques, often aimed at limiting the spread of pests, it will be necessary to implement new methods to ensure an increasingly better result in biological control. The last few years have seen a significant growth of information about the interaction between hosts and parasites; in particular, the interface between parasites and the host immune system has recently attracted much attention from worldwide researchers, mainly for the purpose of understanding the assembly and modulation of host immune responses. Finally, a better knowledge of nematocomplex-insect relationships could provide an improvement in integrated pest management (IPM) techniques and although these nematodes are widely used in the integrated management of agricultural pests, new perspectives are emerging for the use of EPNs in the control of pests and vectors.

The study of the immune response in insects began in relatively recent times, so there is still a long way for a full understanding of the mechanisms underlying the functioning of these processes. This PhD project falls within this topic, and it is intended as the beginning of more in-depth future studies aimed to both a better understanding these mechanisms and improving the conditions of the environment in which we live.

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