Evaluation of tolerance and susceptibility to conventional and biorational insecticides in *Chrysoperla externa* and *C. asoralis* (Neuroptera: Chrysopidae)

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“the top environmental problems are selfishness, greed and apathy…

… and to deal with those we need a spiritual and cultural transformation.”

Gus Speth

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Table of Contents

List of abbreviations

a.i.: active ingredient

Colony B: colony from a field treated with applications of azadirachtin.

CHP: Horticultal Belt of La Plata (*Cinturón Hortícola Platense*)

DEF: S,S,S-tributyl phosphorotrithioate (esterase enzyme synergist).

GC-ECD: Gas Chromatography – Electron Capture Detector

IGR: Insect growth regulator.

IPM: Integrated Pest Management.

MFRC: Maximum field recommended concentration

P450: P450 mono-oxygenase enzymes.

PBO: piperonyl butoxide (mono-oxygenase P450 enzyme synergist).

Colony PN: colony from a field treated with applications of pyrethroids and neonicotinoids.

CHAPTER I

GENERAL INTRODUCTION

I.1. Agricultural pests and pesticides

From the very beginnings of agriculture some 10,000 years ago, man has had to deal with those crop damages produced by pests (Devine *et al*. 2008). According to the world Food and Agriculture Organization (FAO 2000), a pest is: *whatever species, strain, vegetal or animal biotype, or pathogen that is damaging to plants or vegetal products*.

One of the methods of pest control most widely used throughout history has been, and continues to be, the use of pesticides, either natural or synthetic (Devine *et al*. 2008). According to the U. S. Environmental-Protection Agency (EPA), a pesticide is any substance or admixture of substances destined to prevent, destroy, or control any pest whatsoever—including vectors of human or animal illness (*i. e.*, zoonotic diseases); the unwanted species of plants or animals that cause damage or interfere in any form with the production, processing, storage, transport, or commercialization of foodstuffs, agricultural products, and wood, as well as those by-products for animals—or anything that can be administered to animals to combat insects, arachnoids, or other pest in or on the animals' bodies.

The EPA considers that the term *pesticide* includes substances formulated for use as plant growth regulators, defoliants, desiccants, agents that reduce fructification density or prevent premature fruit detachment as well as those substances applied to crops, either before or after harvest, to protect the product during storage and transport. Fertilizers, animal or vegetal nutrients, food additives, and medicines for animals are considered not to fall within this rubric.

I.1.1. Problems associated with the use of pesticides

Although the use of pesticides has played a significant role in agriculture and still continues to be one of the most common practices at the present time, the extensive utilization of those compounds has occasioned a plethora of adverse effects (Naumann 2000; Norris *et al*. 2003; Stark & Banks 2003; Desneux *et al.* 2007; Pórfido *et al.* 2014), such as:

- A reduction in the populations of beneficial organisms (*e. g.*, the natural enemies of pests and pollinator species), causing a disruption of the natural biological balance, as a resurgence of the original pestilence along with secondary occurrences;
- A resistance to those pesticides of the same phytophagous target organisms;
- A chemical contamination of water, the soil, and the atmosphere;
- A disequilibration of the geochemical cycles;
- A reduction in biodiversity;
- A chemical contamination throughout the trophic chain through bioaccumulation and biomagnification;
- An exposure of human populations to those same pesticides through consumption of products contaminated with residues of those compounds that can even become concentrated through biological magnification. In this regard, uncontrolled aerial applications of pesticides through crop spraying near populated areas are the source of major human-health risks.

I.2. Conventional pesticides

Different types of pesticides are currently in use, with the first in commercialization having been compounds with a broad spectrum of activity. Those pesticides constitute a heterogeneous group with divergent mechanisms of action and varied chemical characteristics. As such, the compounds act on a wide range of pest species, but also with the ability to affect non-target organisms as well (Devine *et al*. 2008).

The broad-spectrum pesticides appeared on the market from the decade of the '40s and from that time on have produced an all-encompassing change in the pest-management scenario. The first pesticide to be commercialized was dichlorodiphenyltrichloroethane (DDT), initially used at that time to combat the malaria vector (*i. e.*, the *Anopheles* mosquitoes carrying the parasitic protozoan *Plasmodium falciparum*); but owing to its wide spectrum of action, DDT continued to be used for the control of agricultural and urban pests. Other compounds of broad spectra of activity that have been used both widely and massively are the organophosphates Malathion™, methamidophos, and chlorpyrifos along with the carbamates aldicarb and carbofuran (Smith *et al.* 2002).

The pyrethroids are a group of broad-spectrum pesticides isolated from pyrethrum, a mixture of six natural esters—the pyrethrins—obtained from the flowers of *Chrysanthemum* sp. (that genus originally known as *Pyrethrum*). Although the first pyrethrin analogues were synthesized in the decade of the '40s—with, among them, allethrin exhibiting notable insecticidal activity—the most significant developments in

pyrethroid syntheses occurred in the '60s from the laboratory of Elliot and colleagues (Elliott 1977).

The pyrethroids are pesticides that interfere with neuronal transmission, with their site of action being the voltage-dependent sodium channels (Wakeling *et al*. 2012). When the compounds bind to the target site, they prolong the period during which the sodium channels remain open, thus causing an accumulation of the cation within the neuron that prevents nerve re-polarization. This effect generates a hyperexcitation and a subsequent blockage of the electrical impulse that causes a paralysis, postration, and death of the insect (Sfara *et al.* 2006; Soderlund 2012; 2015).

Among these pyrethroids, the action of cypermethrin (Fig. I.1)—first synthesized in 1974 and whose empirical formula is $C_{22}H_{19}Cl_2NO_3$—was evaluated in the present research.

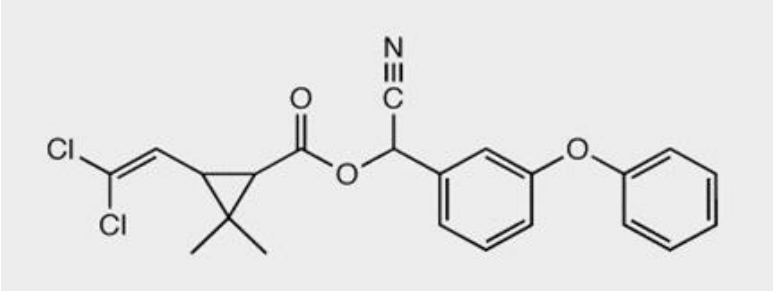


Fig. I.1. Chemical structure of cypermethrin

I.2.1. The use of conventional pesticides in Argentina

Argentina is an agroproducing country whose primary economy is based on crops of soybeans, maize, wheat, sunflowers, peanuts, seed and citrus fruits, fodder,

vegetables, cotton, tobacco, pitted fruits, sugar cane, rice, grapes and grape products, and beans, among others (CASAFE 2015).

As of the '40s decade, the organochlorine pesticides have been widely used in both Argentina and throughout the world. During that same period, the organophosphate and carbamate pesticides stormed the market. From the '70s on, the production and utilization of such conventional pesticides increased in Argentina and other grain-producing countries. Very gradually other classes of compounds, such as the pyrethroids, became incorporated into the market (Brunstein *et al*. 2009).

Owing to the negative consequences occasioned by the indiscriminate use of conventional pesticides, the Argentine National Service of Vegetal Health and Agricultural Foodstuffs (*i. e.*, the Servicio Nacional de Sanidad Vegetal y Calidad Agroalimentaria [SENASA])—the entity responsible for the registration of agrochemicals in the country—through the employment of different resolutions, has restricted or prohibited the use of certain organochlorine, organophosphate, and carbamate pesticides (Pórfido *et al.* 2014). However, around 1000 active ingredients and 3000 registered formulations are in the national market, with variable toxicities and environmental risks. This requires of a great caution when used, instead of an indiscriminate manner. Additionally, Argentina is one of the countries that produce a high percentage of the active ingredients that consumes (17%, after China) (Fig. I.2) (Pórfido *et al.* 2014).

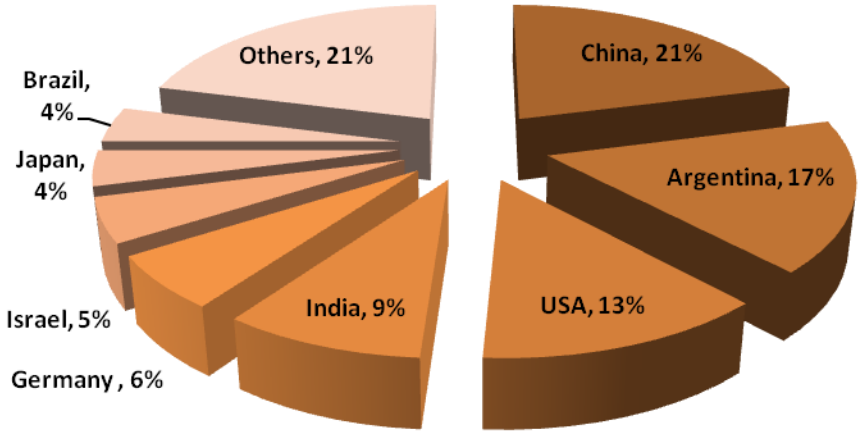


Fig I.2. Countries of origin of phytosanitary products (de Pórfido *et al.* 2014)

That Argentina is the principal exporter of soybean oil and flour, and the third provider of oleaginous grains worldwide, is indeed notable (FAO 2013). The principal pesticides currently in use in this country are, first and foremost, the herbicides glyphosate and atrazine—in treatments of large expanses of soybean and transgenic-maize crops—followed by cypermethrin, lambda-cyhalothrin, and chlorpyrifos (Villaamil Lepori *et al*. 2013). In the Horticultural Belt of La Plata, the most important products are tomato, sweet-pepper, aubergine, which are produced in greenhouses. The harvest season is the one with high temperatures of the year (from the end of August until April) and in this moment the phytosanitary products are applied, most of the times in a systematic and preventive manner (Capello & Fortunato 2008).

I.3. Integrated Pest Management

Integrated Pest Management of (IPM) is defined as a system of decision-making for the selection of strategies of pest control that are harmonious: this approach is

accordingly based on the analysis of cost *versus* benefit while taking into account the interests of various sectors and the overall impact on the producers, the society, and the environment (Kogan 1998; Radcliffe *et al*. 2009).

Within the context of IPM, a pest is whatever organism increases its population enough to reach the level of economic damage, defined as the population density of a phytophagous species, which can cause economical damage (Alston 1996). When a phytophagous population attains the level of economic damage, the cost associated with the management of the pest equals the economic benefit of such control. Pest populations below that level do not create a need to resort to any measure of control. The economic-threshold level is a population density somewhat lower than the level in which the cost and the damage done on the value of the product, equals the treatment cost (i.e. when the relation between the cost of control and obtained benefit is a bit lower than 1:1 (Naranjo *et al.* 2015). In such an instance, some manner of pest control should, however, be initiated in order to prevent such a threshold population from reaching the level of economic damage (Fiedler *et al*. 2008; Maleki & Damavandian 2015).

The different strategies implemented for IPM include control through agriculture (*e. g.*, mechanical agronomic methods, soil management, and crop rotation), phytogenesis (*e. g.*, the use of genetically improved pest-resistant cultivar strains), ecology (*e. g.*, control by autocides, consisting in the introduction of sufficient numbers of artificially sterilized individuals into a population to halt reproduction; the use of sexual pheromones to attract massive numbers of the appropriate insects into lethal traps, and the application of repellents); chemistry (*e. g.*, insecticides, herbicides, and pesticides); and biology (*e. g.*, predation and infection of pests) (Van Driesche *et al*.

2007). This dissertation will focus on the latter two approaches. Although the principles of IPM establish a preference for biological and agricultural control over the use of chemicals; nonetheless, when resorting to the latter form of pest management becomes unavoidable, biorational insecticides constitute the agents of choice (Kogan 1998).

I.3.1. Biorational insecticides

Biorational insecticides are compounds—natural or synthetic—derived from microorganisms, plants, or minerals. These agents, being selective for specific pests, generally possess a single mechanism of action and are both compatible with beneficial organisms and of low environmental impact. In addition, biorational insecticides exhibit an extremely low degree of toxicity for humans and other vertebrates (Ishaaya & Horowitz 2009).

As indicated in the following section, biorational insecticides fall into different groups depending on their target site and mechanism of action: insect-growth regulators (IGR), bioinsecticides (botanical insecticides, microbial insecticides), synthetic molecules with new modes of action, etc. Below, the products used in this work will be detailed.

I.3.1.i. Insect-growth regulators

Insecticides that regulate insect growth are synthetic compounds that intervene in the processes of insect reproduction and/or development. This group includes the synthetic analogues of the insect juvenile hormone, those that mimic the steroidal insect-moulting hormone ecdysone (Carlson *et al.* 2001), and chitin-synthesis inhibitors (Dhadialla *et al*. 2010).

The processes of development and moulting in insects are regulated by a complex hormonal interaction involving both the juvenile hormone and ecdysone wherein the relative concentrations of those two humoral agents within the insect body determine the stages in those processes. Ecdysone, secreted by the ecdysial glands, favors the process of metamorphosis; while the juvenile hormone, elaborated by the *corpora allata*, a pair of endocrine glands located behind the brain, interacts with ecdysone to regulate ecdysis, the latter defined as the shedding of the external cuticular layer of the insect body. During the larval stage, the juvenile hormone is liberated, whose function is to maintain the immature developmental characteristics of the organism. Accordingly, low concentrations of that hormone allow moulting to occur (Hoffmann & Porchet 2012).

Pyriproxyfen (Fig. I.3)—of empirical formula $C_{20}H_{19}NO_3$—is an insecticide commonly used in Argentina that mimics the action of the juvenile hormone (Pórfido *et al.* 2014) and whose insecticidal activity occurs upon either contact or ingestion. Once inside an organism, the active principle functions as a juvenile-hormone agonist so as to interfere with the development, growth, and vitellogenesis of the target insect (Sullivan & Goh 2008).

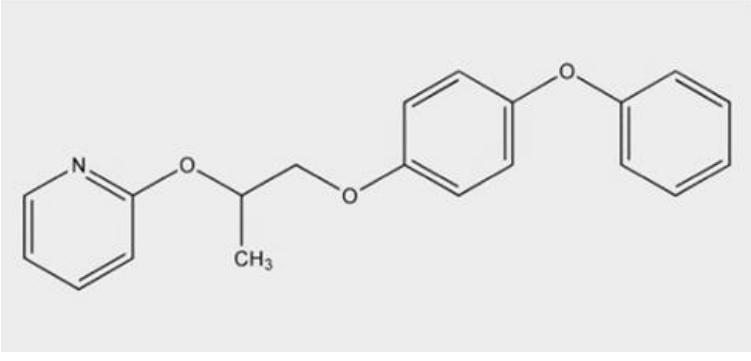


Fig. I.3. Chemical structure of pyriproxyfen

I.3.1.ii. Botanical pesticides

The botanical pesticides—natural or synthetic compounds that either are or function as secondary metabolites utilized by the plants in defense against phytophagous organisms—represent adaptations in plants acquired during their co-evolution with herbivorous insects (Isman 2006; 2008).

The botanical insecticide best known worldwide and most extensively utilized in Argentina is azadirachtin (Fig. I.4) (Pórfido *et al.* 2014). The active ingredient in this compound is a tetranorterpenoid of the limonoid class extracted from the seeds of the Neem tree or Indian lilac (*Azadirachta indica* A. Juss.) (Sapindales, Meliaceae). The names azadirachtin A, B, C and D (azadirachtin C is no longer used as it was not described), have been proposed for different compounds belonging to different chemical groups, but all from the neem extract. Later on, other compounds (E, F and G) were isolated (Morgan 2009). These substances can repel insects; prevent their installation; or interfere with their feeding behavior (an antifeedant effect), growth, development, and reproduction. The effect on feeding occurs when the insecticide stimulates the chemoreceptors in the insect tarsi, mouthparts, and oral cavity. In other instances, azadirachtin acts as an ecdysone antagonist to block the binding sites of the hormone, thus interfering with the moulting process (Mordue 2004).

Though azadirachtin—with empirical formula $C_{35}H_{44}O_{16}$—exhibits a low toxicity to vertebrates, its action on the natural enemies of pests and on pollinating organisms still remains controversial (Barbosa *et al.* 2015).

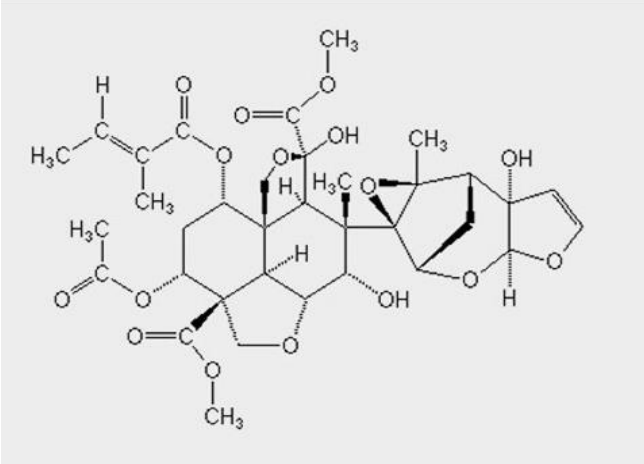


Fig. I.4. Chemical structure of azadirachtin

I.3.1.iii. Neonicotinoids

The neonicotinoids are compounds whose principal activity can be likened to that of nicotine. Imidacloprid was the first member of this class of compounds to be commercialized from the beginning of the '90s. The rapid implementation in the market of these insecticides is attributable to certain of their properties such as the ability to control a broad spectrum of arthropod pests, their different mechanism of action from those of the conventional pesticides, and—most significantly—a low toxicity to mammals (Goulson 2013).

The neonicotinoids act selectively and irreversibly on nicotinic acetylcholine receptors in the cholinergic neurons of the insects, first paralyzing then finally killing them (Tomizawa & Casida 2005).

Another neonicotinoid insecticide commonly used in Argentina is acetamiprid (Fig. I.5), of empirical formula $C_{10}H_{11}ClN_4$ (Pórfido *et al.* 2014).

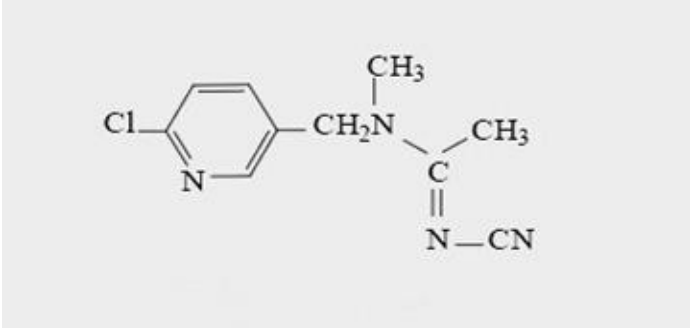


Fig. I.5. Chemical structure of acetamiprid

Though the neonicotinoids have been considered as biorational insecticides for more than a decade now, the overall toxicological categorization of these compounds remains quite controversial, principally because of their proven high toxicity to pollinating insects and pest predators (Desneux *et al*. 2007; Goulson 2013).

I.4. Resistance, tolerance, susceptibility

Resistance to pesticides is the capacity of a pest population to tolerate doses of toxic agents that would otherwise prove lethal to the majority of the individuals of the same species (Stenersen 2004). Every application of a pesticide has a drastic effect on the population of target insects. The individuals that survive such applications carry chromosomal alleles conferring drug resistance that upon those insects' reproduction become more frequent within the population. Because of this possibility, certain pesticides exhibit a restricted period of usefulness since the onset of resistance on the part of the pests would render those compounds ineffective.

In the present dissertation, the following concepts will be taking into account: resistance, tolerance and susceptibility. Resistance induction (phenomenon at a molecular level where mutant resistant genes are detected), must be distinguished from

a natural tolerance that certain pest species may exhibit. In those instances, a physiological property renders the pesticide ineffective to a majority of the normal individuals (Stenersen 2004). It is a natural tendency of a species, or even of a particular developmental stage, to survive insecticides applications (Coles & Dryden 2014). In contrast, if an organism (developmental stage, species or population) is negatively affected by an insecticide, the organism is susceptible. The susceptibility can be evaluated with mortality records, biological parameters (developmental time, fecundity, fertility, etc.), comparing lethal doses (DL50) between populations, or other protocols (Fathian *et al*. 2015; Grigg-McGuffin *et al*. 2015; Pessoa *et al*. 2015).

The intensive use of pesticides has caused the development of resistance in many species of insects and mites throughout the world. According to the Arthropod Pesticide-Resistance Database (APRD, www.pesticideresistance.org), more than 550 arthropod species have developed such resistance (Whalon *et al*. 2008), which drug-tolerant strains include examples of pests of agricultural and urban impact as well as vectors of plant disease. Moreover, in contrast to the circumstance with pest arthropods, only few examples have been registered of such resistance in the natural enemies of those pests (Pree *et al*. 1989; Pathan *et al*. 2008; Sayyed *et al*. 2010; Mansoor *et al*. 2013; Rodrigues *et al*. 2013). The causes of the more rapid drug resistance in phytophagous arthropods compared to their natural enemies have been attributed to biological, biochemical, and ecological factors (Pathan *et al*. 2008).

The resistance mechanisms are extremely varied and as such are related to the modes of action in the different groups of pesticides.

I.4.1. Resistance to pyrethroids

For many pests, the most prevalent mechanism of resistance to pyrethroids is an insensitivity of the target site, referred to as *knockdown* resistance, caused by point mutations in the gene encoding the voltage-dependent sodium channel. Numerous investigations have been carried out on this type of resistance in mosquitoes (*e. g.*, *Anopheles gambiae* Giles, *An. arabiensis* Patton, and *Aedes aegypti* L.), flies (*Musca domestica* L.), and cockroaches (*Blattella germanica* L.) (Osborne & Smallcombe 2013, Al-Deeb 2014, Gholizadeh *et al*. 2014, Ndiath *et al*. 2014; 2015, Pang *et al*. 2015).

The principal mechanism of arthropod detoxification is mediated by the activity of cytochrome P450, which hemoprotein is the terminal oxidase in electron-transport chains. Less central, but still relevant, is the activity of the xenobiosis-detoxification enzyme glutathione S-transferase, which catalizes the conjugation of the tripeptide glutathione to molecular species destined for degradation. Accordingly, mutations have been identified that either generate an overexpression of that enzyme (*i. e.*, regulatory-gene mutations) or produce changes in the amino-acid sequence (*i. e.*, structural-gene mutations) that result in an increase in the enzyme's catalytic efficiency (Joußen *et al*. 2012; Zhong *et al*. 2013; Edi *et al*. 2014). A reduction in the penetration of the pesticide is also a means of pest drug resistance (Lin *et al*. 2012). In this regard, the degree of resistance that certain pests have manifested against different nicotinamides has demonstrated the adaptive potential of certain organisms to that group of insecticides. The majority of the instances of resistance formation have occurred against imidacloprid (Nauen *et al*. 2013; Bass *et al.* 2015). The introduction of new molecules with the same mechanism of action (such as acetamiprid, thiamethoxam, nitenpiran, thiacloprid, and

clothianidin) would appear to have augmented the exposure of those target arthropods to neonicotinoids and at the same time favored the conditions conducive to the development of resistant phenotypes.

I.5. Biological control

Biological control is achieved through the use of organism populations (natural enemies) for maintaining a population density of a phytophagic arthropod, below the level of economic damage, either temporally or permanently (Van Driesche *et al.* 2007). It is an economical method and "environmental friendly". Depending on the case, natural enemies populations are released to cause a permanent change in the food webs; in other cases it is not expected the reproduction of the natural enemy. This approach is based on the already existing relationship—*e. g.*, predator-prey, host-parasitoid, host-entomopathogen—between different species within an agroecosystem (Eilenberg *et al*. 2001).

The organisms utilized for biological control can be entomophages or entomopathogens (Hajek 2004). The entomophages are divided in predators and parasitoids, with specific characteristics regarding feeding habits, behavior, etc. Predators are insects or arachnids (mites and spiders), generally with a larger size than the prey. When the size is small, the organism uses other strategies as poison injection to the prey. To localize the prey, they use chemical signs, and vision or other stimuli when the prey is nearby. Predators use their mandibles to cut and break the prey body, or they develop tubular structures that insert in the body to suck the fluids. On the other hand, parasitoids are the second major group of entomophages used in pest control. Immature

stages of this organisms, parasite other insects, while the adults are free-living, and they spread out in order to reproduce and search healthy hosts for their progeny. Parasitoids includes a lot of different insect groups: Hymenoptera (65 thousand species), Diptera, Coleoptera, Lepidoptera and Neuroptera.

Entomopathogens are microorganisms that invade and reproduce inside an insect, and spread to infect other insects. These infectious agents could be virus, bacteria, fungi, protists, or even multicelular animals (nematodes). Infected insects exhibit some synthoms, like aberrations or dysfunction, which are characteristic from the desease (Vega & Kaya 2012).

Agroecosystems contain complex networks of organisms that interact with each other, and these interactions are structured by the relative rhythm of biological and ecological events. Land management intensification and global climate change, threaten to unpairing the temporal structure of the interaction networks, and disrupt the supply of ecosystem services such as biological control. Therefore, it is critical to recognize the central role of these temporal dynamics to boost the predator-prey interactions in an agroecosystem. Specifically, the population dynamics in cultures behave as periodic oscillations, or cycles (Welch & Harwood 2014).

Biological control can be applied by any of the following strategies: *Classical Biological Control* was the first one to be used on a large scale, and is based on the introduction of an exotic natural enemy species of the pest species. Regulatory programs of this strategy have been widely used with success (Eilenberg *et al*. 2001; Van Driesche *et al*. 2007; Myrick *et al*. 2014).

The *Neoclassical Biological Control* involves the introduction of an exotic natural enemy species of the pest species to reduce a native pest. This encounter results in a

new predator-prey interaction since the pest and the beneficial organism have not coevolved. Neoclassical biological control is a useful strategy when the natural enemies associated with a pest within its native habitat fail in imposing an effective control over it. In such instances, the control agent of choice is one that has successfully regulated similar pest in other areas (Eilenberg *et al*. 2001; Van Driesche *et al*. 2007).

Other strategy of control is the *Augmentative Biological Control,* which consists in increasing the number of natural enemies already present within the agro-environment— possibly even through various releasing events of those antagonistic organisms to control a pest. In this approach, two types of releases can be used: inundative and inoculative (Eilenberg *et al*. 2001; Van Driesche *et al*. 2007). a) *Inundative Biological Control* implies the release of large amounts of individuals of the biocontrol agent, to accomplish pest control in a short term. Accordingly, the continued reproduction of the beneficial organism is never expected. Inundative releases must therefore be repeated whenever the pest species manages to restore its population damaging levels. The released agents must be capable of controlling a sufficiently great proportion of the pest population—or at least of reducing the phytophagous numbers below the level of economic damage (Eilenberg *et al*. 2001; Van Driesche *et al*. 2007). b) *Inoculative Biological Control* is the release of the beneficial agent, and it is expected to replicate to an extent sufficient for that generation as well as its progeny to control the pest at hand. This strategy accordingly provides a pest control that is self-sustainable over the long term, in contrast to the biological control by inundation. To accomplish control success, the release must involve a sufficient number of individuals for self-establishment within the environment, so as to thus maintain a second or third generation present for continued biological control. In addition, the environmental

conditions must favor the organism's multiplication—*i. e.*, through the availability of a wide diversity of crops, sites for refuge from predators, and alternative prey and/or hosts (Eilenberg *et al*. 2001; Van Driesche *et al*. 2007).

Finally, the *Biological Control by Conservation* is based on the protection of a pest's natural enemies. It was originally developed to recover the action of beneficial organisms that had been adversely affected by the application of synthetic pesticides. This strategy involves practices that protect populations of a pest's natural enemies or contribute to their replication (*i. e.*, through the provision of sites of refuge, alternative prey, and other food resources). This approach therefore promotes the development of biodiversity and enhances the trophic relationships among organisms (Eilenberg *et al*. 2001; Van Driesche *et al*. 2007).

This type of biological control should be an important alternative against the indiscriminate use of pesticides as well as a significant component of sustainable agriculture. The manipulation of the environment (habitat) of natural enemies must be directed to their higher survival, physiological and behavioral performance, and must result in an improved efficiency as agents of control (Barbosa 1998). The presence of the natural enemy in the area before the control takes place and, therefore, the previous adaptation to the environment of the pest is an important aspect of biological control by conservation. This last point is relevant in this thesis, as discussed in the following sections.

I.5.1. Natural enemies

In this approach, a pest's natural enemies—those being taxonomically diverse and consisting of predators, parasitoids, and pathogens (see Section I.5)—are utilized to control an arthropod pest.

For a pest's natural enemy to be considered successful, the latter must possess certain attributes in combination: (1) a specificity for the host or prey, (2) a growth synchronization with the pest, (3) a high replication rate, (4) a habitat in which to survive periods with little or no presence of the phytophagous organism, and (5) a proficient hunting capability (Hajek 2004).

This work was focused in one of the natural enemies groups, the predators. Some examples of predators employed in biological control programs are: arachnid species of the order Acarina—*e. g.*, *Phytoseiulus persimilis* Athias-Henriot, *Amblyseius cucumeris* Oudemans, and *Neoseiulus cucumeri* Oudemans (Van Driesche *et al*. 2007); the coccinellids *Harmonia axyridis* Pallas, *Hippodamia convergens* Guérin-Méneville*, Eriopis connexa* Germar (Hodek *et al.* 2012); hemíptera of the families Geocoridae, *Geocoris punctipes* Say and Anthocoridae, *Orius insidiosus* Say (Wong & Frank 2013); predatory members of the order Diptera within the families Syrphidae—*e. g.*, *Phacelia tanacetifolia* Benth and *Episyrphus balteatus* DeGeer—and Cecidomyiidae—*e. g.*, *Aphidoletes aphidimyza* Rondani and *Dasineura rubiformis* Koselik (Van Driesche *et al*. 2007); and the chrysopids *Chrysoperla carnea* Stephens, *C. rufilabris* Burmeister, and *C. externa* Hagen (van Lenteren 2012; Flores *et al.* 2013; Lavagnini *et al*. 2015).

The present dissertation will be focused on Neuropterans, their presence in agroecosystems and the effects of insecticides on them.

I.5.2. Neuropterans

The neuropterans (Table I.1) are holometabolous insects. The adults have orthognathous or hypognathous heads, and mouthparts evolved for chewing, with strong jaws, though these features vary among species. The antennae are filiform or moniliform, and the individuals possess large compound eyes usually in the absence of ocelli. The prothorax is short and the legs usually thin and cursory—though in some instances are short and robust—while the two pairs of large wings are membranous with a complex venation. The abdomen is in 10 segments, but 9 in the Chrysopidae. The larvae are campodeiform and prognathous—*i. e.*, with a forward projecting mouthpart. The jaws and maxillae on each side are strongly fused forming a suction tube, with the maxillary palpi being absent (New 2001).

Table I.1. Taxonomy of the species studied in the present thesis.

PHYLUM: Arthropoda

SUBPHYLUM: Hexapoda

CLASS: Insecta

SUBCLASS: Pterygota

ORDER: Neuroptera

SUBORDER: Hemerobiiformia

FAMILY: Chrysopidae

SUBFAMILY: Chrysopinae

GENUS: *Chrysoperla*

SPECIES: *Chrysoperla externa, Chrysoperla asoralis*

The Chrysopidae are the most diverse family of the order Neuroptera. Eggs are cylindrical and present colour changes during insect embryogenesis. The newly laid brightly green eggs darkened significantly to a grey-brownish colour. Unfertilized eggs remained light green. The eggs have a button-like micropylar process at one ápex and at the opposite end a short, thin, flexible stalk fastens the eggs to the substrate. The micropylar process has a central canal for the passage of sperm and consists of porous material, which serves the embryo in gas exchange.

The larvae of the chrysopids (three larval stages in total) are polyphagous—and even commonly cannibalistic—predators with the latter behavior being particularly accentuated in the more advanced larval stages (New 2001). Those larvae have elongated and interconnected mandibles and maxillae that form suctorial tubes as long as their cephalic parts and curved inwards. The buccal aperture is nonfunctional and remains physically covered by the integument. At the base of the mandibles are secretory glands elaborating salivary enzymes that are injected into the prey to initiate the preoral digestion before sucking out the latter's bodily fluids (Canard 2001). The digestive tube is closed off at the juncture of the middle intestine, rendering the portion posterior to that point nonfunctional. The soluble products of the digestion are carried forward in the hemolymph and finally excreted through the Malpighian tubules. The insoluble products are stored in the region distal to the intestine in the immature larval stages, but ejected in the adult along with the imaginal ecdysis.

The third larval stage starts the pupation building a protective silk that enclosed the decticous pupae, and it is formed by numerous layers of white-green fibers of different thickness firmly stuck together at points of contact. The resulted cocoon is

ovoid in shape. It is believed that important differences in the composition of the pupal cocoon may exist between species.

The chrysopid adults have a diet that has been erroneously classified as phytophagous as opposed to the more correct category of *glucophagic*. Accordingly, those imagines feed on nectar and various vegetal exudates along with pollen and the honey-like products of herbivorous insects (Lundgren 2009).

The genus *Chrysoperla* Steinmann is the most appropriate one for the use of specimens as agents of biological control in IPM programs. *Chrysoperla carnea* Stephens is the most commercialized species within this family (Duelli 2001). Some authors consider the *carnea* as a single morphologic species—it being widely distributed within the Holarctic Region, while others believe that this species is more correctly represented by a group of cryptic species all having similar characteristics. This so-called *megaspecies* is therefore more accurately denoted by the term "*carnea* group" (Tauber & Tauber 1973; Brooks 1994; Henry *et al.* 2001).

I.5.2.1. Chrysopids in Argentina

The following 4 species of the genus *Chrysoperla* have been registered in Argentina: *C. externa* Hagen, *C. asoralis* Banks, *C. argentina* González Olazo & Reguilón, and *C. defreitasi* Brooks (Monserrat & de Freitas 2005) (Fig. I.6).

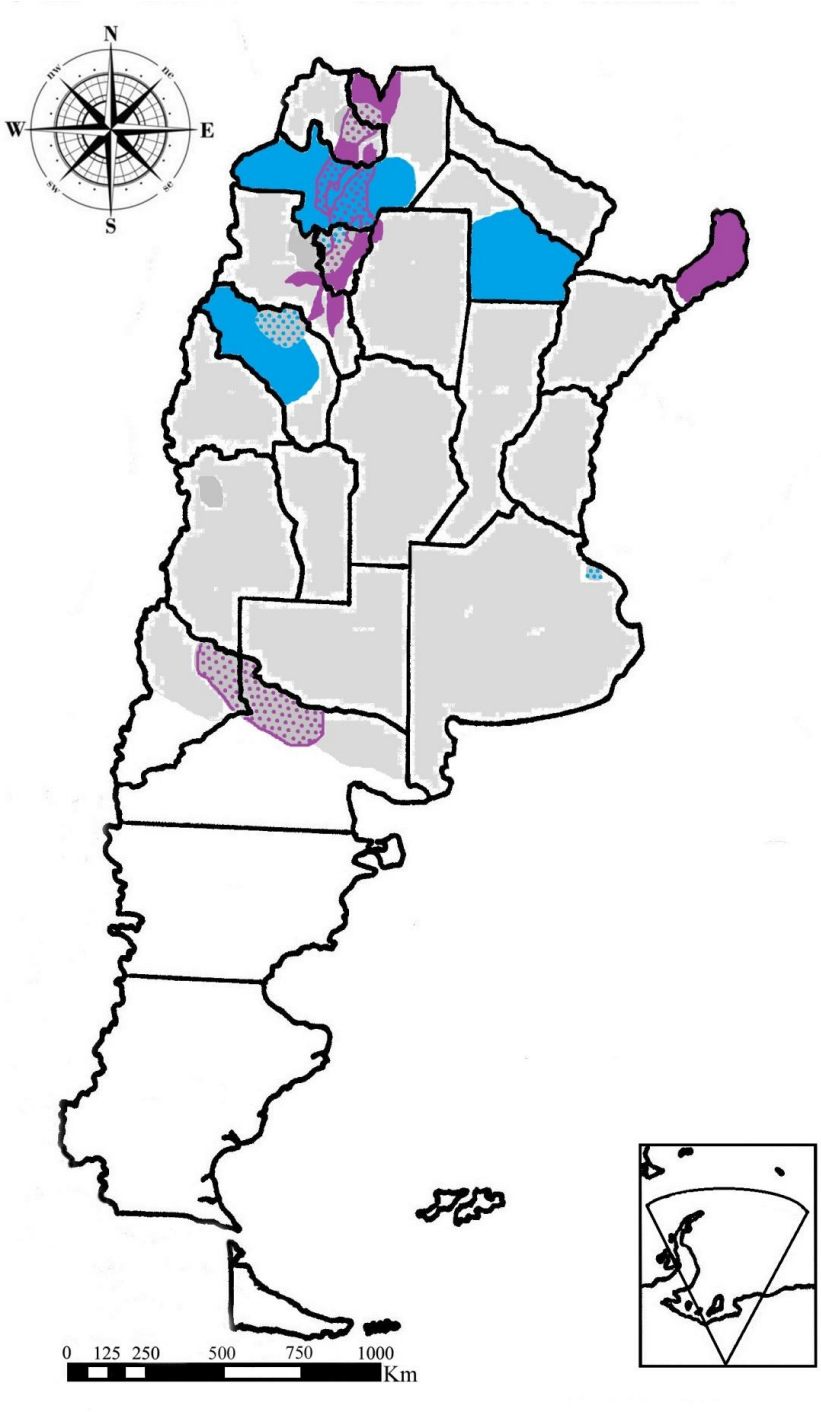


Fig. I.6. *Chrysoperla* distribution in Argentina. *C. argentina* (dark gray), *C. asoralis* (blue), *C. defreitasi* (violet) and *C. externa* (light gray).

Chrysoperla externa is widely distributed in the Neotropical Region from the Antilles in the Caribbean down to the north of the Patagonia in Argentina (Adams &

Penny 1985); *C. asoralis* is found from the north of Argentina to the north of the Patagonia (González *et al.* 2011; González Olazo & Heredia 2007); *C. argentina* has been registered in the Argentine provinces of Salta, Chaco, La Rioja, and Tucumán (González Olazo & Reguilón 2002; Reguilón *et al*. 2006); while *C. defreitasi* manifests a much narrower distribution, it being restricted exclusively to the ecoregion of the Yungas in eastern Argentina (González *et al*. 2011) and to the north of the Patagonia (Monserrat & de Freitas 2005).

Of these 4 species, *C. externa* has been the most extensively studied with investigations on different aspects of its biology and its susceptibility to pesticides having been carried out (Fig. I.7) (Iannacone & Lamas 2002; Silva *et al*. 2006; Rimoldi *et al*. 2008; Moura *et al*. 2011; Rimoldi *et al*. 2012; Schneider *et al*. 2009). Their massive rearing has been promoted in South America (Salamanca Bastidas *et al*. 2010), but only in Peru it is reared in biofabrics for its release (SENASA Perú 2015).

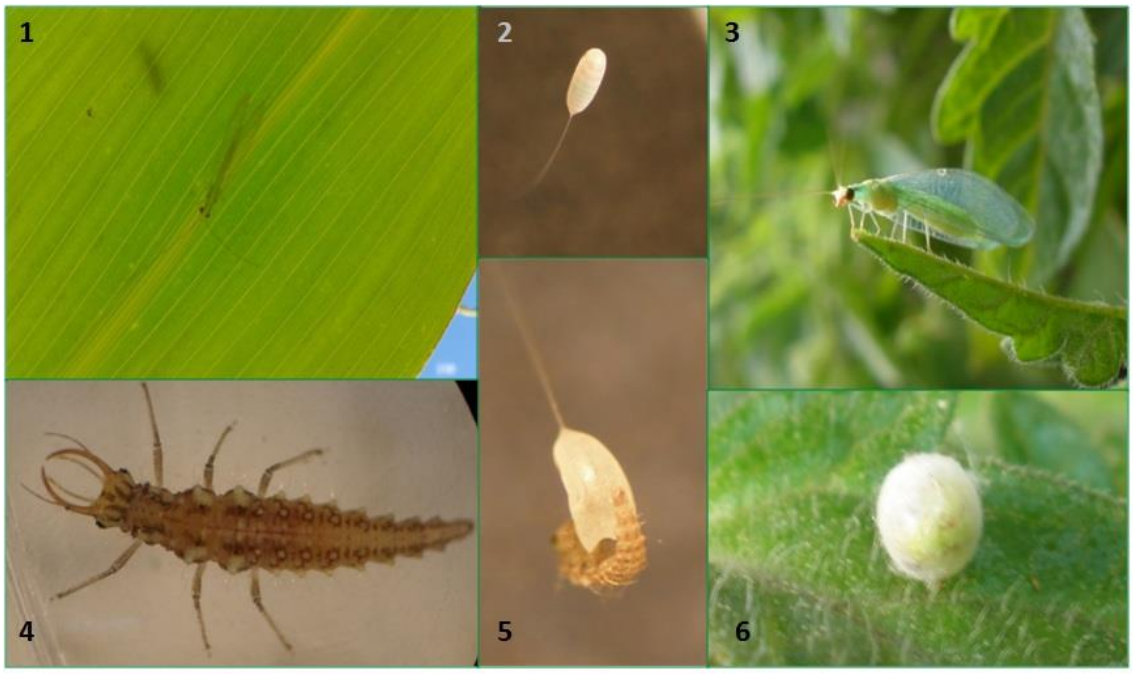


Fig. I.7. Development stages of *Chrysoperla externa*. Egg (2), larvae (4,5), pupa (6), adult (1,3).

On the other hand, *C. asoralis* has been registered in Argentina in a much smaller area and in areas geographically distant from each other. This species is an important predator of various agricultural pests, mostly in fruit crops, and is distinguished from *C. externa* for its larval and adult morphological characteristics (Chapter II). It has been described the morphology of the different stages of the species (González Olazo *et al*. 2009) and there is some information on the toxicity of certain insecticides (a carbamate and one botanical insecticide) in Peru (Iannacone *et al.* 2015).

Because of the presence and importance as biological agents of the species and *C. externa* and *C. asoralis*, the applications of insecticides in the Horticultural Belt of La Plata (CHP) and the premises of IPM, which point out the use of natural enemies and ultimately combine this strategy with the use of selective insecticides, the following thesis addresses the toxicity of different insecticides on these two species. This will be of importance for future studies, as it provides data on mortality and on how the biological parameters of beneficial species are affected with the use of these chemicals.

According to González *et al.* (2011), *C. asoralis* could be displacing *C. externa* on the basis of the high number of individuals found in several locations. Although more studies in the field have to be carried out in order to corroborate the hypothesis, an initial collect done during a two-year period indicated that *C. asoralis* was more abundant than *C. externa*. The presence of this species was registered in an IPM crop with low frequence of biorational insecticides applications; this could be due for a higher susceptibility to conventional pesticides in *C. asoralis* than in *C. externa*. For this reason, comparative studies of the susceptibility in both species were carried out.

I.6. General objective

The objective of the present thesis was to evaluate the susceptibility, tolerance or resistance, of *C. externa* and *C. asoralis* to different pesticides. Toxicity bioassays were performed analyzing the lethal and sub-lethal effects, and studies at molecular level in laboratory and field colonies, as well. The presence of *Chrysoperla* species in the Horticultural Belt of La Plata (Buenos Aires province, Argentina) was also studied. The subsequent susceptibility evaluation of *C. asoralis* and *C. externa* allowed the increase of the knowledge of tolerance aspects in both species.

I.6.1. Specific objectives

1. To study the presence of different specimens of *Chrysoperla* spp. in the Horticultural Belt of La Plata.

2. To evaluate the susceptibility in laboratory and field colonies of *C. externa*, exposed to cypermtehrin, acetamiprid and azadirachtin, through biological parameters (survival rate, developmental time, preoviposition period, fecundity and fertility)

3. To evaluate the resistance in laboratory and field colonies of *C. externa*, exposed to cypermethrin, through molecular studies, searching of resistant DNA sequences, enzymatic detoxification mechanisms, and residues analysis of the insecticide.

4. To compare the susceptibility of *C. externa* and *C. asoralis* to cypermethrin, acetamiprid and pyriproxiphen.

I.7. Hypotheses

The first objective was descriptive.

For the second and third objective, the following hypothesis was formulated:

- Individuals from field populations of *C. externa* have developed resistance mechanisms to cypermethrin, acetamiprid and azadirachtin, compared with laboratory individuals. Field populations were exposed to insecticide applications for several generations.

Finally, the hypothesis associated with the fourth objective was:

- *C. externa* presents a higher tolerance to cypermethrin, acetamiprid and pyriproxiphen compared with *C. asoralis*, because the latter was only collected in IPM crops, without exposure to broad-spectrum insecticides.

CHAPTER II

GENERAL

MATERIALS AND METHODS

II.1. Field collecting

The collecting of crysopids in the field was carried out in different commercial crops of the Horticultural Belt of La Plata (CHP) (Fig. II.1). These crops were (*Solanum lycopersicum* L.), sweet-pepper (*Capsicum annuum* L.), aubergine (*Solanum melongena* L.) and peas (*Pisum sativum* L.), in greenhouses. Besides, spontaneous grass near the crops, were sampled.

The sampling was made manually and with entomological nets. Adults, eggs and larvae were the stages collected, and they were sampled weekly from the end of spring until summer (2011 – 2014). The objective of the collecting was to maintain the *Chrysoperla* colonies in the laboratory.

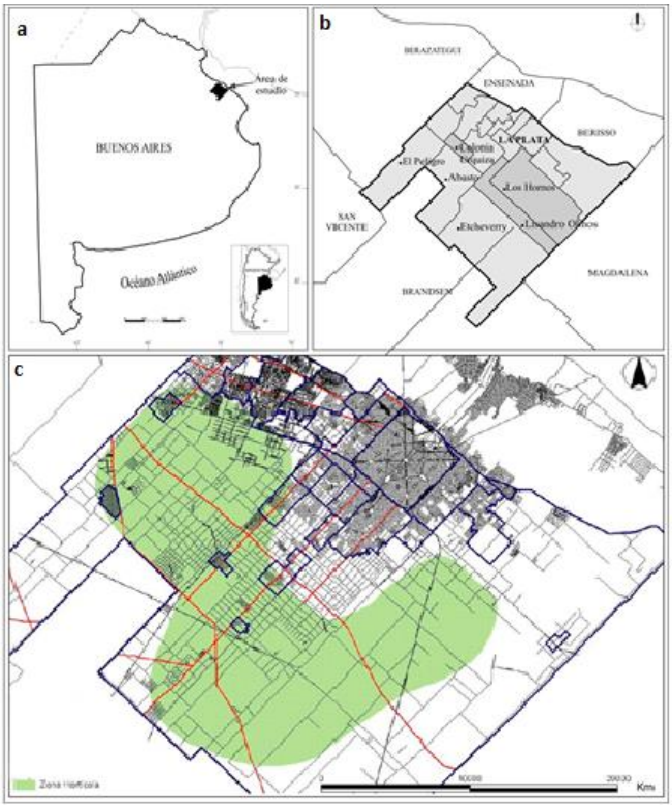


Fig. II.1. a) La Plata District in Buenos Aires Province; b) different localities in La Plata District (Colonia Urquiza, Los Hornos, Abasto, Lisandro Olmos, Etcheverry y El Peligro); **c)** La Plata region with the Horticultural Belt in green (Source: Ringuelet 2008 in Rouaux 2015).

II.1.2.i. Chrysopids colonies: collections and rearing of laboratory organisms

In this dissertation different colonies of the predator *C. externa* were established (Fig. II.2).

- The laboratory colony was composed by specimens reared for various generations and without exposure to pesticides. The laboratory of Ecotoxicology of CEPAVE (Center of Parasitological and Vectors Studies), had a laboratory colony of *C. externa* since 2006, without exposure to pesticides. To avoid inbreeding, new organisms from crops without applications were added annually. Individuals were collected in the experimental station "Julio Hirschhorn" (Faculty of Agricultural and Forestry Sciences, UNLP) and maintained in quarantine to avoid the development of diseases. This colony was used as "susceptible colony" to be able to compare it with the field colonies.

- The field colony named PN (pyrethroids and neonicotinoids), were represented by the progeny of collected individuals from crops with a long history of pesticide applications, such as pyrethroids (cypermethrin, deltamethrin, lambda-cyhalothrin) and neonicotinoids (imidacloprid, thiamethoxam and acetamiprid). The georeference was: 34°91'58,46'' S, 58°02'22,11'' O.

- The field colony named B (botanical), were composed by the progeny of individuals from crops with "organic treatment", with periodical applications of the insecticide azadirachtin. The georeference was: 34°94'37,19'' S, 58°12'99,69'' O.

Besides, a laboratory colony of *C. asoralis* was reared. The former was represented by specimens without exposure to pesticides. The georeference was: 34°90'65,70'' S, 58°14'25,70'' O.

The colonies were placed in plastic jars of 15 cm height. Adult were fed on artificial diet (Vogt *et al*. 2000) and drinking devices of 10 ml of volume, were provided with distilled water. Black cardboards were provided to each jar to facilitate the extraction of the eggs.

Larvae were fed on *Rhopalosiphum padi* L. (Hemiptera: Aphididae) and with artificial diet as a supplement. Once in the third larval stage they were individualized in plastic ventilated capsules, of 2 cm diameter and 1 cm height, to avoid cannibalism. After the pupal period and when the adults emerged, couples were established by the external genitalia with a binocular loupe.

II.1.2.ii. Rearing and maintaining of *R. padi* colony

The initial colony of *R. padi* was provided by Ing. Agr. Mónica Ricci. Wheat plants were growth (ACA901 variety) as the host plant. Seeds were embedded in water for 48 h minimum to promote budding, and then were placed in little plant holders. These plants were placed in ventilated jars of 40 cm long, 30 cm width And 25 cm height. Wheat seeds were provided by Ing. Agr. Carlos Bainotti (INTA Marcos Juárez) and Ing. Agr. Carlos Junquera.

II.1.2.iii. Rearing conditions and bioassays

Insect rearing and bioassays were done under controlled conditions of temperature (25 ± 2 °C), relative humidity (70 ± 5%) and photoperiod (16:8 L:D). These conditions were possible with a split for the temperature regulation, a humidifier and a timer, all of this checked with a digital thermometer/hygrometer.

II.2. Experimental design

Bioassays on chrysopids species consisted on the applications of pesticides, considering different development stages and by topical application. Solutions were prepared taking into account the Maximum Field Recommended Concentrations (MFRC) of each insecticide. Active ingredients, commercial names, purity, MFRC and supplier, are indicated in Table II.1.

Table II.1. Insecticides used in the experiments.

Active ingredient	Commercial name	Purity (w/v)	MFRC (mg a.i./L)	Supplier
Cypermethrin	Glextrin®	25%	25	GLEBA S.A.
Acetamiprid	Mospilan®	20%	200	SUMMIT AGRO
Pyriproxyfen	Epingle®	10%	75	SUMMIT AGRO
Azadirachtin	Neem-Azal®	1,2%	40	AGRISTAR S.A.

The application was topical on the dorsal part of the abdomen in the larvae, and on the cocoon in the pupae, with a manual micro-applicator (Hamilton®, Switzerland). A droplet of 1 µl was applied. The solutions were prepared with acetone analytical grade, and control only with acetone.

II.3. Statistics

In general, One-way Analysis of Variance (ANOVA) was used, with previous normality and homoscedasticity evaluations by Shapiro-Wilk and Levene tests, respectively. Data (mortality, developmental time, preoviposition period, fecundity and fertility) that were expressed as proportions were transformed by the equation: $y = \sqrt{arcsen\ x}$. When data did not achieve the ANOVA assumptions, the transformations were made with the equation: $y = log\ (x+1)$. Being x, the non transformed data.

Transformed data were again tested. When they did not achieve the assumptions, a non parametric Kruskal-Wallis was used. Means and medians were analyzed by Fisher (LSD) or Dunn tests depending on the analysis (Scheiner 1998). $P < 0.05$ was considered significant. The software used was XLSTATSTART.exe (2014).

Fig. II.2: Pictures of collecting fields, rearing and feeding of chrysopids. In the bottom left margin, Hamilton® micro-applicator.

CHAPTER III

FIRST RECORD OF *CHRYSOPERLA ASORALIS* AND *C. ARGENTINA* (NEUROPTERA: CHRYSOPIDAE) IN HORTICULTURAL FIELDS OF LA PLATA ASSOCIATED WITH SWEET PEPPER (*CAPSICUM ANNUUM* L.)

1. Introduction

The green lacewings— considered to be highly efficient predators— are used for the biological control of various pests, such as aphids, coccids, thrips, and lepidopteran larvae (Lingren *et al*. 1968; Canard *et al*. 1984; Greeve 1984; Thompson 1992; Bento *et al*. 1997; Hamilton & Lashomb 1997; Urbaneja *et al*. 1999). In Argentina, the following four species of *Chrysoperla* Steimann, 1964 have been recorded: *C. externa* (Hagen 1861), *C. asoralis* (Banks 1915), *C. argentina* (González Olazo & Reguilón 2002) and *C. defreitasi* (Brooks 1994) (Montserrat & de Freitas 2005). *Chrysoperla externa* shows a broad distribution in the Neotropical region: in Argentina it is found from the northernmost provinces down to the north of Patagonia (Adams & Penny 1985); *C. asoralis* is likewise present from northern Argentina to northern Patagonia (González Olazo & Heredia 2007; González *et al*. 2011); *C. argentina*, has thus found only in the provinces of Salta, Chaco, La Rioja and Tucumán (González Olazo & Reguilón 2002; Reguilón *et al*. 2006); while *C. defreitasi* has been recorded exclusively in the forests of the Yungas region in eastern Argentina (González *et al*. 2011) and in northern Patagonia (Montserrat & de Freitas 2005). The actual distribution of *C. argentina* and *C. asoralis* in Argentina, is provided in Fig. III.1, the first one in Chaco, Salta, Tucumán, La Rioja and Buenos Aires Provinces; the second one in Chaco, Salta, Jujuy, Tucumán, La Rioja, Mendoza, Neuquén, Río Negro and Buenos Aires Provinces.

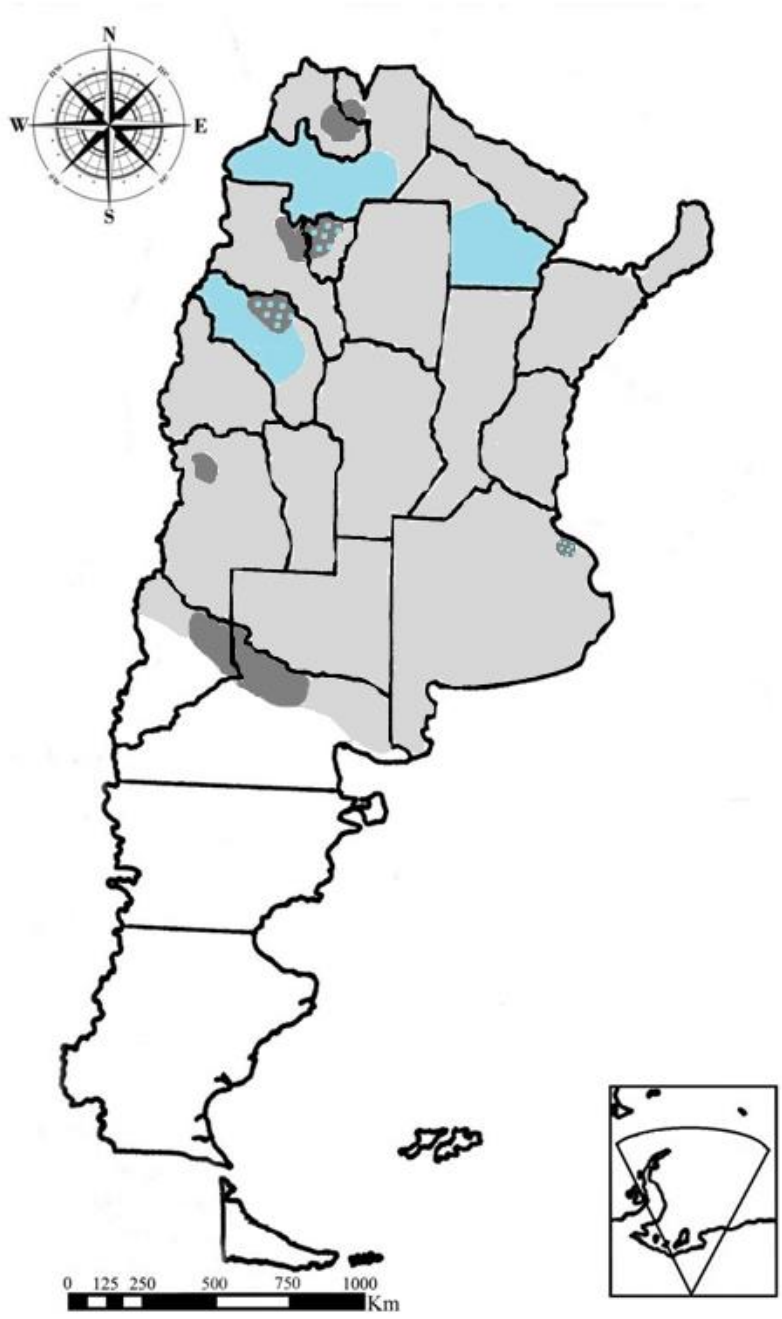


Fig. III.1. Distribution map of *Chrysoperla argentina* (light blue), *C. asoralis* (dark gray) and *C. externa* (light gray) in Argentina. Inset: Argentinian Antarctic Sector.

* Cardinal points were found in: http://sp.depositphotos.com/1075605/stock-illustration-compass-rose.html.

The species most thoroughly investigated is *C. externa*, and different ecotoxicological studies have already been reported (Iannacone & Lamas 2002; Silva *et*

al. 2006; Rimoldi *et al*. 2008, 2012; Schneider *et al*. 2009; Moura *et al*. 2012). Its massive rearing and subsequent release in the field has been promoted in several countries during recent years (Vargas, 1988; Daanel & Yokota 1997; Carvalho *et al.* 2002; Pappas *et al*. 2011).

The Horticultural Belt of La Plata (CHP) is one of the most extensive in the Buenos Aires province, occupying 65% of the greenhouse-cultivated area of the province (Censo Hortiflorícola 2005). The sweet pepper (*Capsicum annuum* L.) is one of the main crops in this region. Several pests attack both the fruit and the plant, such as the green peach aphid *Myzus persicae* (Sulzer) (Barbosa *et al*. 2008) and the whitefly *Bemisia tabaci* (Genadius). Both these pests are of economic significance because of their direct effect on the plant itself through the sucking of the phloem and their secondary role as vectors of viral phytopathogens.

In the horticultural agroecosystems of La Plata, the presence of *C. externa* has been detected in both organic and conventional fields, and in association with these two pests. The objective of the present chapter was registering the different species of *Chrysoperla* that are present in the horticultural zone of La Plata.

2. Materials and methods

2.1. Species collecting

Collecting made in greenhouses with different crops in the area of La Plata, Buenos Aires province (34°90’65.67"S, 58°14’25.71"W), were made in 2012-2013 (spring and summer seasons). Those crops were under an integrated pest

management. Collecting was performed randomly using entomological nets and aspirators.

2.2. Taxonomic identification of Chrysopidae specimens

The species determination was done in the Miguel Lillo Foundation, Institute of Entomology (Tucumán province, Argentina) by Dr. C. Reguilón. Larval stages were preserved in 65% ethanol and adults, dry frozen. For the identification, taxonomic keys were used, based on distinctive characteristics, i.e. cephalic marks in all larvae stages, genae differences, post-ocular marks and crossveins of the anterior wings in adults (González Olazo & Reguilón 2002; Reguilón *et al.* 2006; González Olazo *et al.* 2009).

3. Results

About 60 adults and 35 eggs were collected during three different dates, which F1 was identified as *C. externa*, *C. asoralis* and *C argentina*. The last two species represented the first record for both species in the Buenos Aires province (specifically in CHP), the distribution was extended and in association with sweet pepper crops *C. annuum*, as they were collected only in those ones. The larval stage of *C. asoralis* has a rounded cephalic dorsolateral mark, with two lateral extensions and one anterior extension; parallel and big dorsolateral marks. *C. argentina* has a narrow cephalic dorsolateral mark not bifurcated. *C. externa* has a central anterodorsal mark with bifurcations in the rear part, and big dorsolateral marks bifurcated in the anterior part (Fig. III.2-3). *C. asoralis* adults have two red postocular spots, pronotum without lateral bands and red genae. *C. externa*, have a pronotum with red lateral bands and red

genae. *C. argentina* does not have postocular marks nor dark-brown genae (Fig. III.3-7).

Table III.1: Sampling with number of finded individuals.

Collecting date	**Adults**	**Eggs**
December 2012	37	20
January 2012	23	15
March 2013	1	0

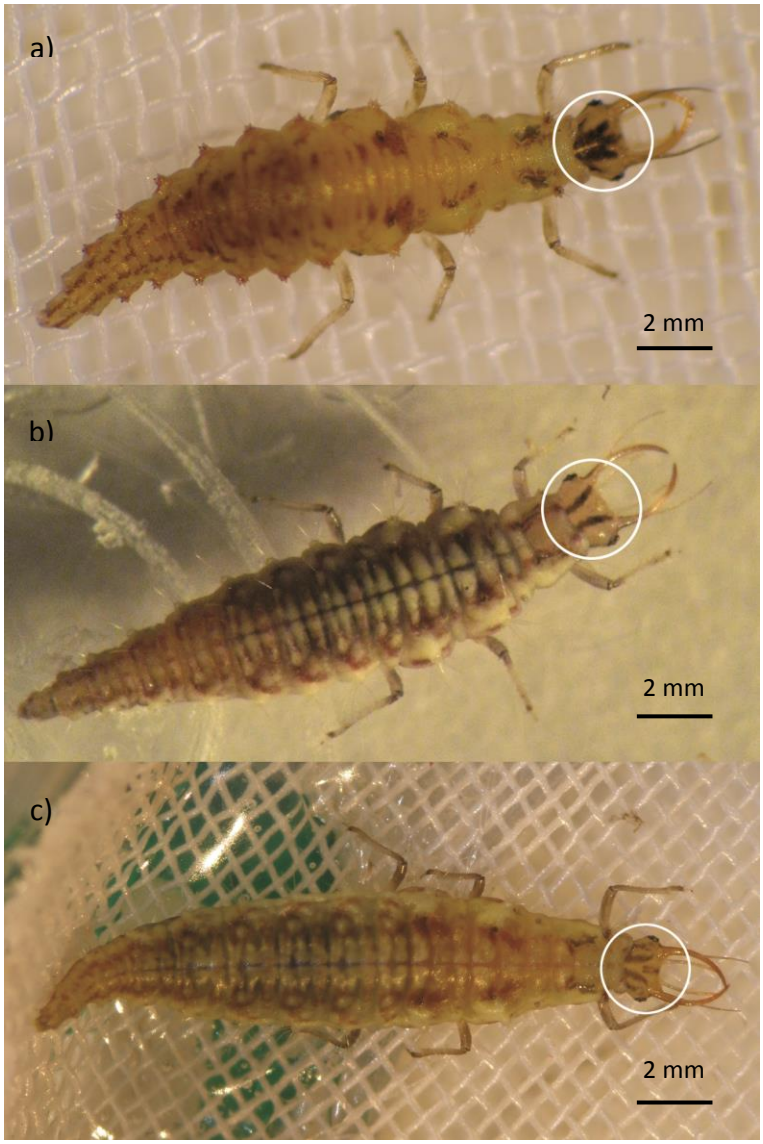


Fig. III.2. Third instar larvae of a) *Chrysoperla asoralis* with large rounded cephalic dorsolateral marks connected with the medium mark; b) *C. argentina* with two narrow cephalic dorsolateral marks; c) *C. externa* with a bifurcated anterodorsal medium mark and irregular dorsolateral marks.

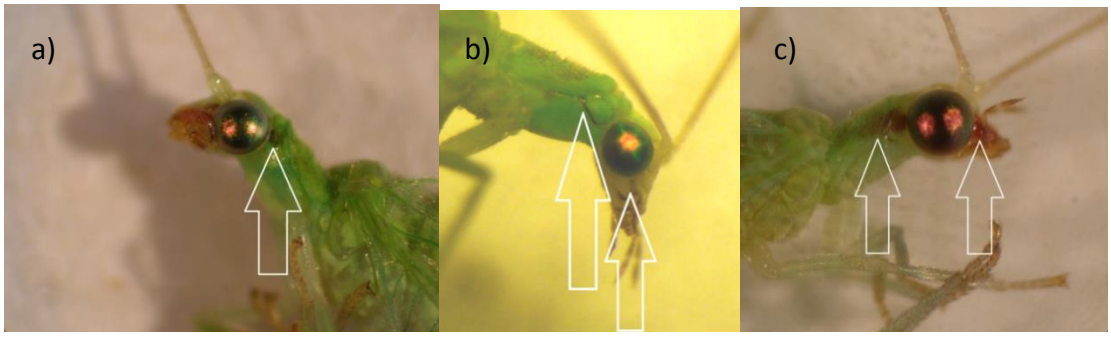


Fig. III.3. Adults with head detail of a) *Chrysoperla asoralis*, red postocular spot, pronotum without lateral bands; b) *C. argentina*, dark brown genae; c) *C. externa*, pronotum with red lateral bands, red genae.

4. Discussion

Novel record of the presence of two *Chrysoperla* species in the Horticultural Belt of La Plata region, information on their distribution (Adams & Penny 1985; Montserrat & de Freitas 2005; Reguilón *et al*. 2006; González Olazo & Heredia 2007; González *et al*. 2011), and a report of the new association with the sweet pepper, is provided in this Chapter. Furthermore, the most relevant taxonomic characters of the larvae and adults of these species are included.

According to González *et al.* (2011), *C. asoralis* could be displacing *C. externa* on the basis of the high number of individuals found in several locations. Although more studies in the field have to be carried out in order to corroborate the hypothesis, an initial collect done during a two-year period indicated that *C. asoralis* was more abundant than *C. externa*. The presence of this species was registered in an IPM crop with low frequence of biorational insecticide applications; this could be due for a higher susceptibility to conventional pesticides in *C. asoralis* than in *C. externa*.

The association of *C. asoralis* and *C. argentina* with sweet-pepper pests would point out this species as being a potential biological control agents, with the potential of being used in IPM protocols. Finally, the present study provided basic information on the taxonomy of the *Chrysoperla* species before their mass rearing for field releases.

CHAPTER IV

TOXICITY OF PESTICIDES IN LABORATORY AND FIELD POPULATIONS OF *CHRYSOPERLA EXTERNA* (NEUROPTERA: CHRYSOPIDAE)

IV.1. Introduction

Biological control and selective pesticides have been proven to be compatible with IPM (Kogan 1998; Galvan *et al.* 2005). Although chemical control should be the final option in an IPM program, several agroecosystems depend on pesticide applications, and for that reason it is essential to assess the risks for lethal and sublethal effects against beneficial organisms (Shinde *et al*. 2009). In this case, *C. externa*. It is necessary to perform risks assessment tests, selectivity assays and modes of use of the insecticides, in order to maximize the compatibility of natural enemies and chemicals (Desneux *et al*. 2007).

The chosen insecticides were selected regarding their frequent use in the sampled fields. The pyrethroid cypermethrin is a broad-spectrum insecticide, with a long-term residuality. It has been used indiscriminately since its synthesis in the '60s, with negative effects as the development of insecticide resistance (Zhong *et al*. 2013). In the '90s, neonicotinoids started to be commercialized, and they were classified as biorational insecticides by US-EPA due to their low toxicity in non-target organisms. However, later studies demonstrated a high toxicity to pollinators, and nowadays the classification is under consideration. The bioinsecticide azadirachtin is biorational, with low toxicity to vertebrates, high selectivity and with short-term residuality; however its mode of action and biosynthesis are not well known (Mordue 2004).

The objective of this chapter was to evaluate the lethal and sublethal effects of cypermethrin, acetamiprid and azadirachtin in field and laboratory populations of *C. externa,* through mortality, developmental time, preoviposition periods, fecundity and fertility.

IV.2. Materials and methods

IV.2.1. Insects

The field colonies were PN and B, as detailed in Chapter II; section II.1.2. The individuals of the colony "PN" were collected in a field with a long history of periodical pesticide applications, pyrethroids (cypermethrin, deltamethrin, lambda-cyhalothrin) and neonicotinoids (imidacloprid, thiamethoxam, acetamiprid). The individuals of the colony "B" were collected in an organic field with periodical applications of azadirachtin.

IV.2.2. Insecticides, preparation of solutions

The insecticides were tested at their respective maximum field recommended concentration (MFRC) and several dilutions of this as prepared from the commercial formulates Glextrin® (25% w/v cypermethrin, Gleba S.A.), Mospilan® (20% w/w acetamiprid, Summit-Agro S.A.) and Neem Azal® (1.2% w/v azadirachtin, Agristar S.A.) (CASAFE 2015) (Table IV.1).

Table IV.1: insecticides used in the assays on the third instar larvae from different *Chrysoperla externa* colonies. * Indicates the MFRC of each insecticide.

Treated colony	Active ingredient	Concentrations (mg a.i./L)	Percentages of MFRC	Terminology used in Results
Laboratory	Cypermethrin	12.5	1/2	Cyper 50
vs.		25*	1/1	Cyper 100
PN		37.5	1.5/1	Cyper 150
Laboratory	Acetamiprid	100	1/2	Acet 50
vs.		200*	1/1	Acet 100
PN		250	1.5/1	Acet 150
Laboratory	Azadirachtin	20	1/2	Aza 50
vs.		40*	1/1	Aza 100
B		60	1.5/1	Aza 150

IV.2.3. Toxicity assays in third instar larvae

For the toxicity evaluation with the different insecticides on *C. externa*, third instar larvae from less than 24 h old, were treated. The mode of application was topical, using a Hamilton® micro-applicator. A droplet of 1 µl of the working solution was applied on the dorsal abdomen of each larva. The dilutions and the control were prepared with acetone analytical grade, with the aim of ensure the fast dry and uniform deposition of the insecticide. Thirty replicates by treatment were analyzed.

Treated larvae were placed in little capsules of 1 cm diameter per 2 cm height. They were controlled in a daily basis in order to feed them on *R. padi* as prey and artificial diet as a complement. Survival rate of each stage was registered.

In those treatments where individuals could complete their life cycle, the adult genre was determined and they were paired, to register fecundity and fertility of females, during the first five days of oviposition. Five pairs (repetitions) by treatment were analyzed. Each pair was placed in a container of 4 cm diameter and 6 cm height, and artificial diet and water were supplied. A black cardboard were put inside the containers to facilitate the extraction and counting of the laid eggs. These cardboards were placed in Petri dishes and followed during 10 consecutive days, to register the larval emergence.

This experiment was carried out under controlled conditions of temperature, humidity and photoperiod (25 ± 2 °C; 70 ± 5%; 16:8 L:D).

IV.2.4. Statistics

Larval and pupal mortality, developmental time, cumulative fecundity and fertility were analyzed using Factorial ANOVA for each insecticide. Factors were *colony* and *concentration of the insecticide.* Normality and homoscedasticity assumptions were previously analyzed with Shapiro-Wilk and Levene tests, respectively. $P < 0.05$ was considered significant. XLSTATSTART.exe (2014) was used.

IV.3. Results

IV.3.1. Cypermethrin

All parameters could be measured with cypermethrin treatment (Table IV.2).

IV.3.1.1. Mortality of larvae

Factors did not show a significant interaction (Table IV.2). The colonies were not different, but the concentrations of cypermethrin caused a significant mortality and the higher one was with cypermethrin at 1.5 times the MFRC (Cyper 150) (Fig. IV.1). A 60% of mortality was recorded with the latter, whereas a 30% was caused by the other concentrations.

IV.3.1.2. Mortality of pupae

Surviving larvae which could pupate, did not show mortality in the pupal stage with any of the cypermethrin concentrations tested (data not shown).

Table IV.2. Factorial ANOVA with different concentrations of cypermethrin.

Factors	*Degrees of freedom*	*F*	*P-Value*
A: Mortality in larval stage			
Colony	1	0.25	0.62
Concentration	2	4.74	0.03
Colony x concentration	2	0.56	0.58
B: Third larva to pupa			
Colony	1	3.53	0.06
Concentration	3	8.26	< 0.001
Colony x concentration	3	1.85	0.13
C: Pupa to adult			
Colony	1	1.82	0.17
Concentration	3	3.37	0.02
Colony x concentration	3	6.09	< 0.001
D: cumulative fecundity			
Colony	1	0.06	0.81
Concentration	1	1.09	0.31
Colony x concentration	1	0.09	0.76
E: cumulative fertility			
Colony	1	0.002	0.98
Concentration	1	0.04	0.85
Colony x concentration	1	0.01	0.93

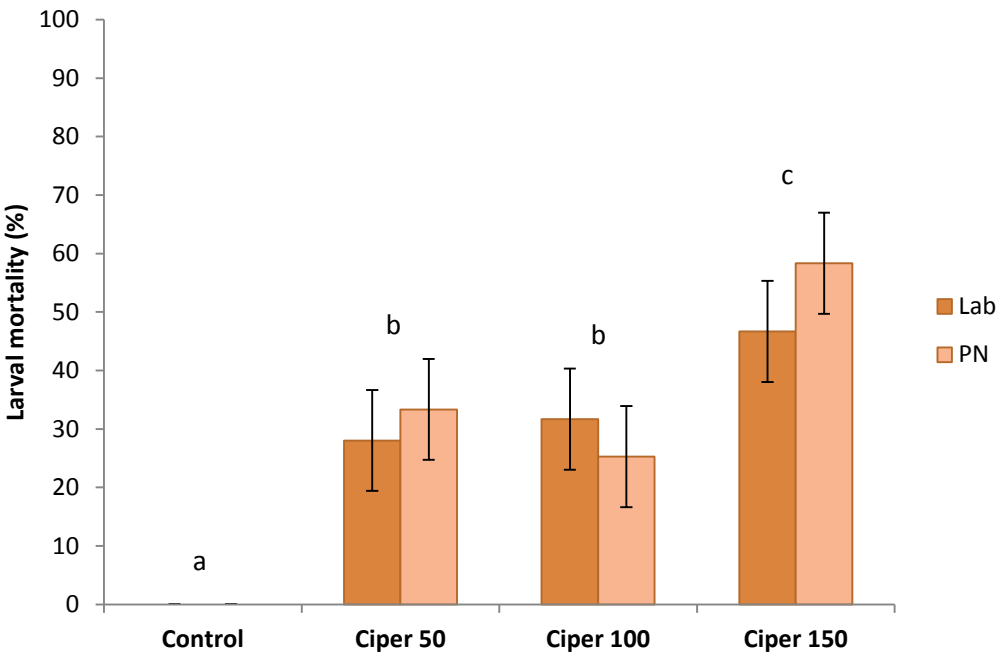


Fig. IV.1. Mortality in larvae of the laboratory and PN colonies treated with cypermethrin. Bars are means ± SE. Different letters denote significant differences between treatments (as there was no differences between colonies, post-hoc tests were made with data from the colonies together) ($P < 0.05$)

IV.3.1.3. Developmental time from larva to pupa

Factors did not show a significant interaction (Table IV.2). The colonies were not different, but all concentrations delayed the developmental time from larva to pupa compared with the control (Fig. IV.2).

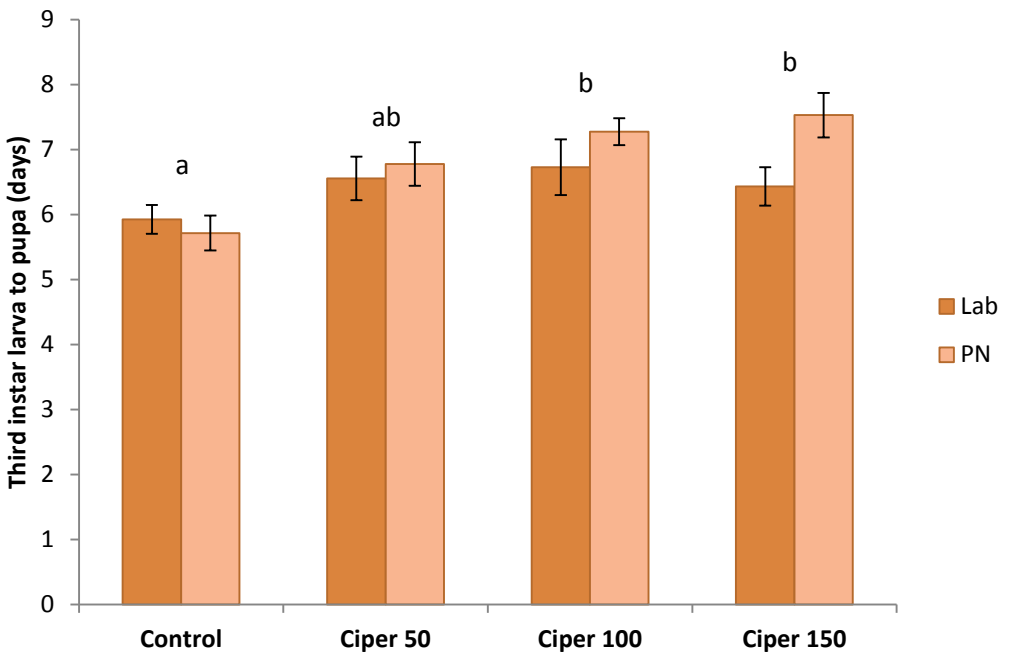


Fig. IV.2: Developmental period from larva to pupa, in the laboratory and PN colonies treated with cypermethrin. Bars are means ± SE. Different letters denote significant differences between treatments (*P < 0.05*)

IV.3.1.4. Developmental time from pupa to adult

Factor colony and concentration had a significant interaction (Table 3), therefore the developmental time of each colony depended of the different concentrations. The developmental time of the laboratory colony was shortened with all concentrations (Fig. IV.3). In the colony PN, this period was longer with cypermethrin at half of the MFRC (Cyper 50), and the other concentrations did not differ from the control.

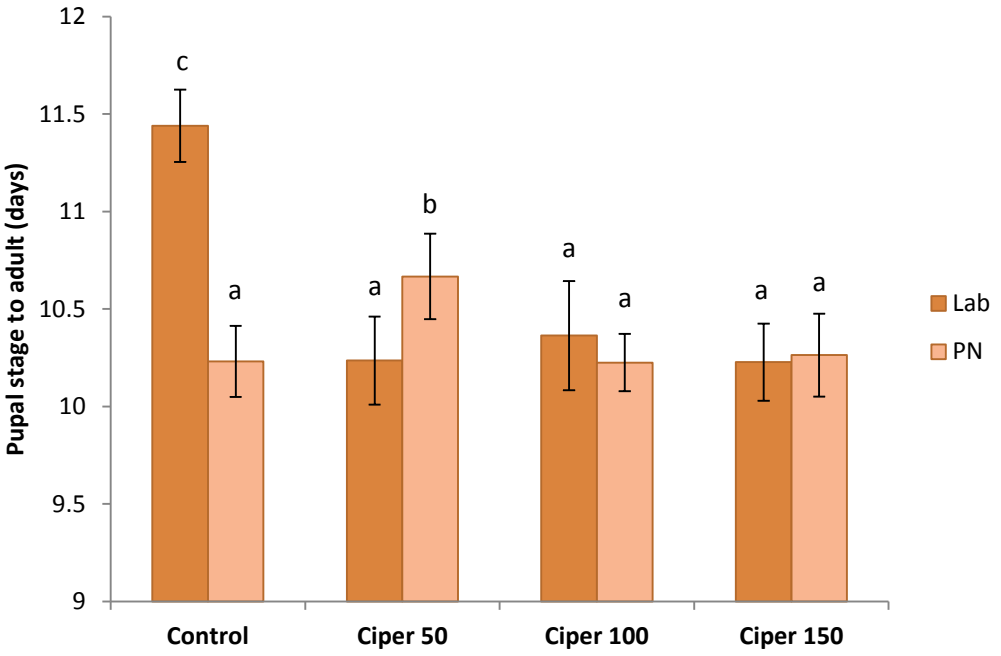


Fig. IV.3: Developmental period from pupa to adult, in the laboratory and PN colonies treated with cypermethrin. Bars are means ± SE. Different letters denote significant differences between treatments (*P < 0.05*)

IV.3.1.5. Reproductive parameters

Factors evaluated in fecundity and fertility did not have a significant interaction (Table IV.2). Cyper 100 did not affect these parameters and colonies were not different between them (Table IV.3).

Table IV.3: fecundity and fertility of females treated with cypermethrin. Data are mean ± SE. Same letters denotes no significant differences between treatments (*P < 0,05*)

Reproductive parameters	Control	Cyper 100
Fecundity (n°eggs/female)	136,8 ± 18,16 a	113,3 ± 11,16 a
Fertility (n°larvae/eggs)	64 ± 0,17 a	63 ± 0,16 a

IV.3.1. Acetamiprid

IV.3.1.1. Mortality of larvae

Factors colony and concentration did not present a significant interaction (Table IV.4). The laboratory and field colonies did not show differences in mortality but this was influenced by concentrations of acetamiprid. As shown in Fig. IV.4, acetamiprid at 1.5 times the MFRC (Acet150) caused a 70% of mortality compared to the control.

Table IV.4: Factorial ANOVA with different concentrations of acetamiprid.

Factors	***Degrees of freedom***	***F***	***P-Value***
A: Mortality in larval stage			
Colony	1	0.55	0.47
Concentration	3	5.03	0.02
Colony x concentration	3	1.13	0.35
B: Mortality in pupal stage			
Colony	1	0.27	0.61
Concentration	3	33.39	< 0.001
Colony x concentration	3	0.59	0.62
C: Third larva to pupa			
Colony	1	3	0.08
Concentration	3	8.75	< 0.001
Colony x concentration	3	1.26	0.28

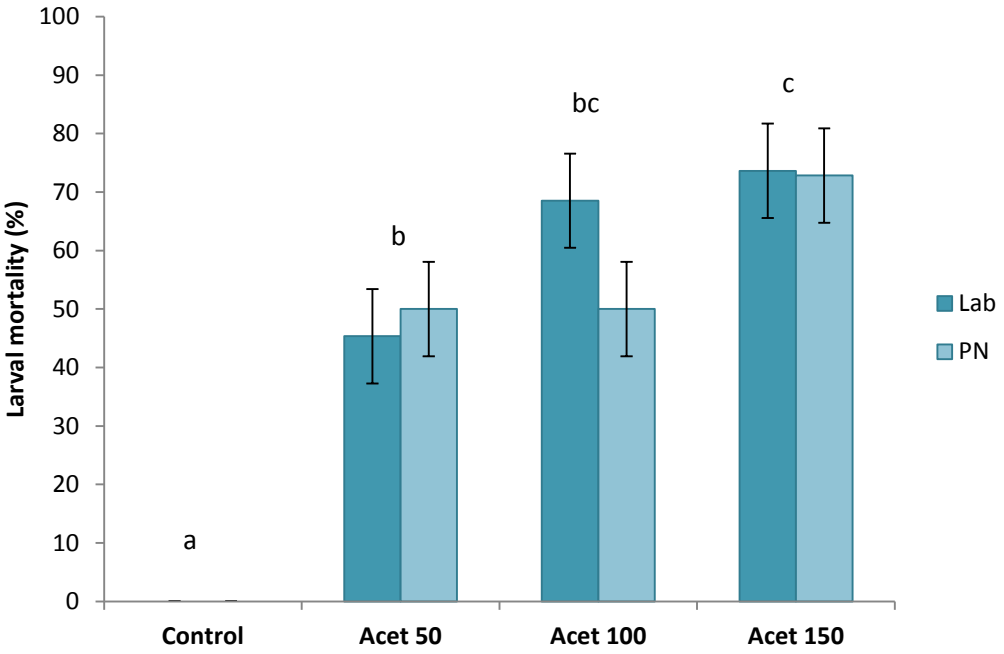


Fig. IV.4: Mortality in larvae of the laboratory and PN colonies treated with acetamiprid. Bars are means ± SE. Different letters denote significant differences between treatments (*P < 0.05*)

IV.3.1.2. Mortality of pupae

The mortality in the pupal stage was not influenced by the colonies, but by the concentrations of acetamiprid (Table IV.4). All the concentrations were different from the control in this developmental stage, and Acet 150 provoked the highest mortality of 50% (Fig. IV.5).

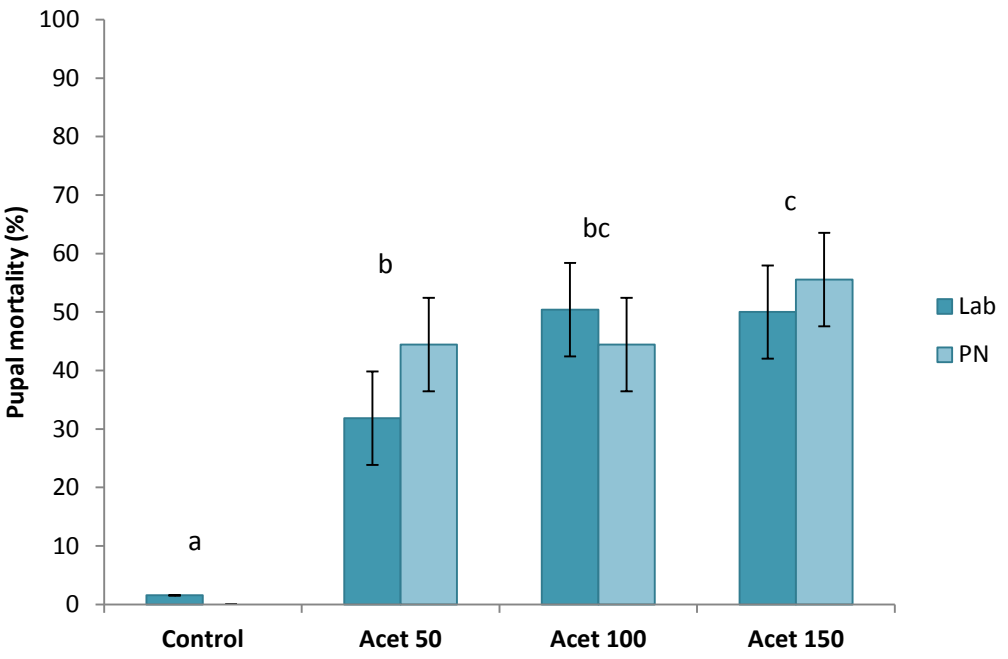


Fig. IV.5: Mortality in pupae of the laboratory and PN colonies treated with acetamiprid. Bars are means ± SE. Different letters denote significant differences between treatments ($P < 0.05$)

IV.3.1.3. Developmental time from larva to pupa

Interaction between factors was not significant (Table IV.4). The development time from larva to pupa was higher with the different concentrations, with a peak in the treatment with acetamiprid at its MFRC (Acet 100) and Acet 150 in the laboratory colony. The colonies did not show differences (Fig. IV.6).

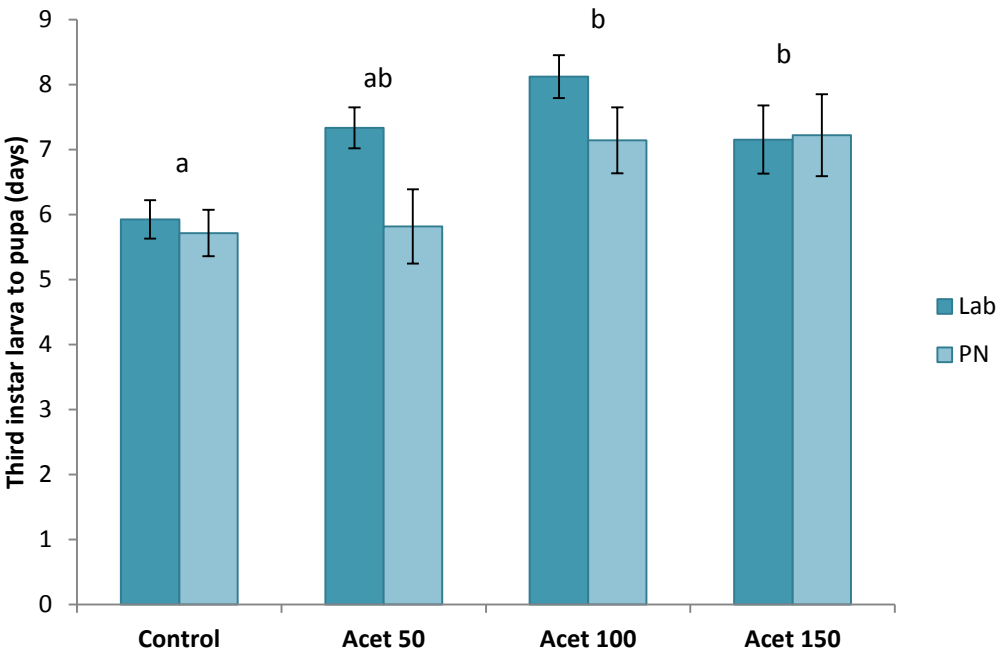


Fig. IV.6: Developmental period from larva to pupa, in the laboratory and PN colonies treated with acetamiprid. Bars are means ± SE. Different letters denote significant differences between treatments. (as there was no differences between colonies, post-hoc tests were made with data from the colonies together) (*P < 0.05*)

IV.3.2.4. Reproductive parameters

Because of the great mortality in the pupal stage, the quantity of adults was not enough to measure the reproductive parameters.

IV.3.3. Azadirachtin

IV.3.3.1. Mortality of larvae

Larvae treated with azadirachtin were not affected in their survival either by concentration and colony (Table IV.5 and Fig. IV.7).

Table IV.5: Factorial ANOVA with different concentrations of azadirachtin.

Factors	*Degrees of freedom*	*F*	*P-Value*
A: Mortality in larval stage			
Colony	1	1.19	0.29
Concentration	3	2.72	0.11
Colony x concentration	3	0.38	0.69
B: Mortality in pupal stage			
Colony	1	0.27	0.61
Concentration	3	5280.23	< 0.001
Colony x concentration	3	0.34	0.85
C: Third larva to pupa			
Colony	1	3.34	0.07
Concentration	3	3.82	0.01
Colony x concentration	3	13.65	< 0.001

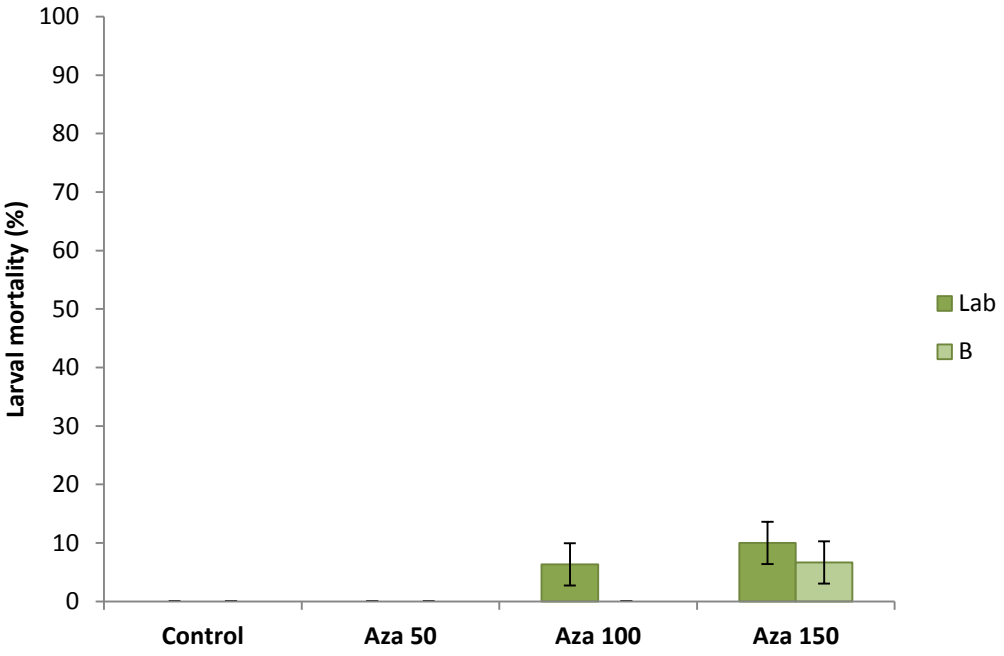


Fig. IV.7: Mortality in larvae of the laboratory and B colonies treated with azadirachtin. Bars are means ± SE. Different letters denote significant differences between treatments (*P < 0.05*)

IV.3.3.2. Mortality of pupae

Factors had not a significant interaction (Table IV.5). Colonies did not differ between each other, and both of them showed 100% of mortality with all the concentrations tested, and a 0% with the control (Fig. IV.8).

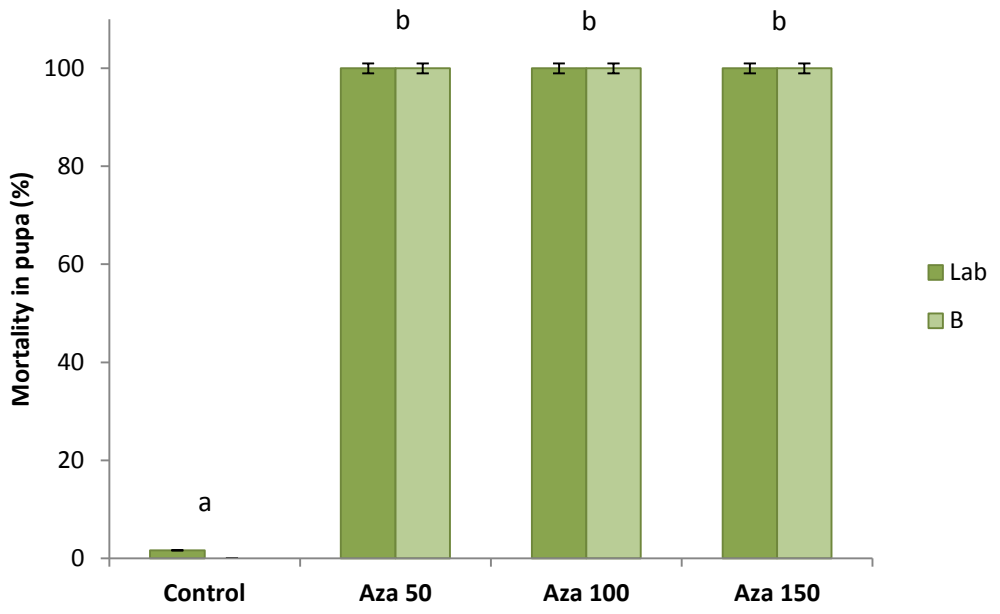


Fig. IV.8: Mortality in pupae of the laboratory and B colonies treated with azadirachtin. Bars are means ± SE. Different letters denote significant differences between treatments ($P < 0.05$)

IV.3.3.3. Developmental time from larva to pupa

Factors colony and concentration had a significant interaction (Table IV.5), the developmental time of each colony depended on the different concentrations. There was a tendency to diminish the developmental time in the laboratory colony, while the concentrations increased (Fig. IV.9). On the other hand, the colony B had a longer period with azadirachtin at 1.5 times the MFRC (Aza 150).

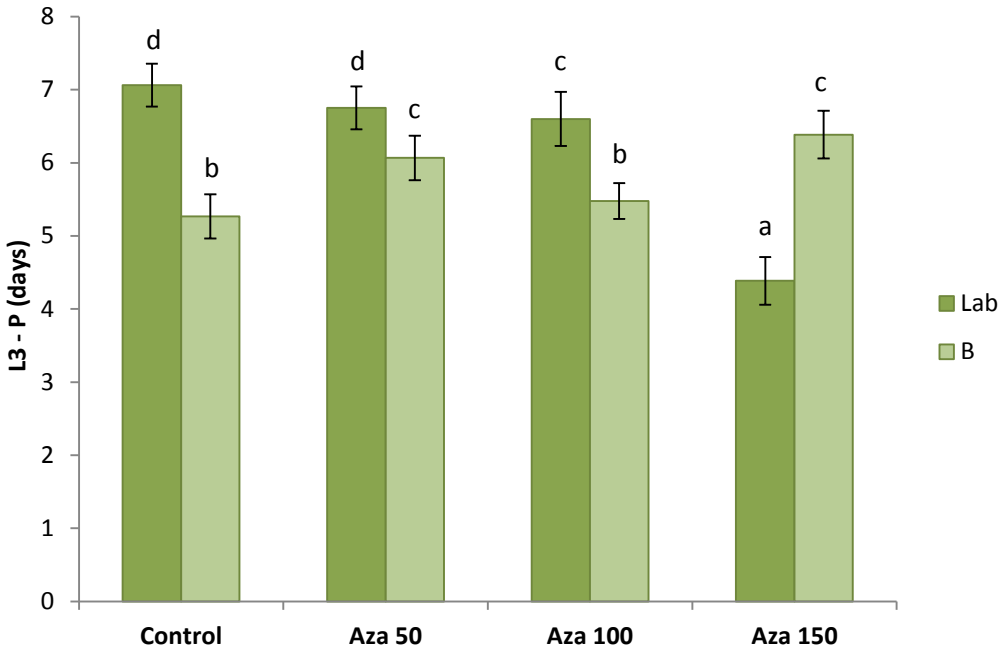


Fig. 9: Developmental period from larva to pupa, in the laboratory and B colonies treated with azadirachtin. Bars are means ± SE. Different letters denote significant differences between treatments (*P < 0.05*)

IV.3.3.4. Reproductive parameters

Because of the great mortality in the pupal stage, the quantity of adults was not enough to measure the reproductive parameters.

IV.4. Discussion

IPM strategies aim to avoid chemical control, and it proposed its use only when all the other methods did not succeed in the pest control (Kogan 1998). The use of biorational insecticides is recommended, which are selective, with a short-term residuality and "environmental friendly" profile (Ishaaya & Horowitz 2009). The agrochemical industry is developing constantly new products for pest management, but the effects of these compounds must be evaluated in natural enemies (predators

and parasitoids) and pollinators, along with testing for pest control efficiency before the commercial release (Desneux *et al.* 2007).

C. externa is considered an efficient predator of agricultural pests and it is used as a biological control agent. Previous studies have shown the tolerance to insecticides in this species, suggesting its suitability as a biocontrol candidate in IPM programs (Moura *et al*. 2010).

The broad-spectrum insecticide cypermethrin has been the object of many studies on chrysopids, mainly in *C. carnea*. The tolerance to pyrethroids of this species has been demonstrated and it was explained by a high enzymatic detoxification. Some authors also speculated with possible resistance at the molecular level, with mutations in the voltage-gated sodium channels, although this was not analyzed in later studies (Bashir & Crowder 1983; Pree *et al*. 1989; Hoy 1990; Pathan *et al.* 2008; 2010; Ishaaya & Casida 1981; Sayyed *et al*. 2010). In the present chapter, the results regarding the tolerance of *C. externa* at MFRC and even higher concentrations of cypermethrin were very clear. It was pointed out that the third larval stage is the most resistant as compared with the first and second larval stages, with 80% of mortality in second larval stage (Sabry & El-Sayed 2011) and 100% in neonates from treated eggs (Rimoldi *et al*. 2008). The pupal stage did not show mortality, and the fecundity and fertility in adults were not different from the control. The laboratory and field colonies had no differences, probably due to an enzymatic detoxification of the pyrethroids during the larval stage. In this sense, *C. externa* could be presenting an elevated detoxification as the Palearctic species *C. carnea*.

The results obtained on the third larval stage of *C. externa* demonstrated a high toxicity by acetamiprid, both in field and laboratory populations. These findings are

consistent with *C. carnea* studies, where the toxicity of several insecticides were evaluated in this species (Vivek *et al*. 2012). Acetamiprid resulted in the most toxic, with 80% mortality after 72 h of treatment, followed by the neonicotinoid thiamethoxam. Shinde *et al*. (2009) observed that the mortality of *C. carnea* exposed to residues of acetamiprid, increased from 53% to 80%, after 24 h and 72 h post-treatment, respectively. In field trials where population parameters *C. carnea* were evaluated in a cotton crop, the treatment with acetamiprid reduced the population of the predator in 36% (Naranjo & Akey 2005).

Other beneficial organisms were evaluated in several studies demonstrating the high toxicity of this insecticide, i.e. the 50% lethal dose (LD50) in the coccinellid *Harmonia axyridis* (Pallas) was much lower than the recommended concentrations for aphids treatments, causing a 100% of mortality in eggs, larvae and adults at doses of 40 mg a.i./L (Youn *et al*. 2003; Awasthi *et al.* 2013). Fogel *et al.* (2013) reported similar results for the predator *Eriopis connexa* (Germar). Other predators as *Orius laevigatus* (Fieber), *Macrolophus caliginosus* (Wagner) and *Amblyseius californicus* (McGregor), were residually affected with the neonicotinoids acetamiprid and thiamethoxam (Van de Veire & Tirry 2003) and the same high toxicity was found for *Podisus maculiventris* (Say) (Tillman & Mullinix 2004).

Regarding the effects on the pupal stage treated with acetamiprid, a reduction of total survival was registered in accordance with the increase of concentration, demonstrating that these effects are maintained during time even more than 10 days after topical treatment. Similar results were shown in *E. connexa* treated with acetamiprid in the fourth larval stage, and the reproductive parameters in the adults from treated larvae were diminished (Fogel *et al*. 2013). This demonstrates the

importance of sub-lethal effects of this insecticide. Negative effects over fecundity were also observed in other predator coleopterans as *Hippodamia undecimnotata* Schneider and *Rodolia cardinalis* Mulsant (Papachristos & Milonas 2008; Grafton-Cardwell & Gu 2003). The supposed harmlessness of acetamiprid, mainly explained by its systemic activity that diminishes the residual contact (Tomizawa & Casida 2005), is not supported by these studies.

The bioinsecticide azadirachtin was found highly toxic to third larval instar. In *C. carnea* larvae studies, azadirachtin applied by ingestion produced lower mortality (Vivek *et al*. 2012), while topical applications caused negative effects only in higher doses (Medina *et al*. 2003). The toxicity of azadirachtin in pests has been widely studied, with mortalities of 100% in lepidopteran larvae of *Pericallia ricini* (Fabricius) (Gnanamani & Dhanasekaran 2013).

The pupal mortality reached 100%, a result also reported in *C. carnea* after topical applications on the third larval stage (Medina *et al*. 2003). Larvae could complete the pupation, but mortality occurred inside the cocoon and in some cases the development went forward but without a complete ecdysis. Vogt *et al.* (1998) reported that a residual application of azadirachtin in larvae, stopped pupation. Azadirachtin affects the growth and development in insects, because it alters the hormonal balance which regulates these processes, *i.e.* by reducing the concentration of ecdysteroids (Morgan 2009). Lepidopteran larvae treated with this bioinsecticide presented longer periods of larval and pupal development, and individuals that could not build the complete cocoon, developed in individuals with characteristics from both stages (the so called “mosaics”) and do not survive (Wondafrash *et al*. 2012).

In the present chapter, individuals of different populations of *C. externa* did not show differences in susceptibility. Thus, it can be suggested that an enzymatic detoxification process is implied and that is not modified by the lack of exposure to insecticides. This species could be characterized as naturally tolerant to toxics. However, susceptibility assays with azadirachtin demonstrated negative effects and resulted as the most toxic insecticide treated. This demonstrates once again the importance of toxicity studies in beneficial organisms that are naturally present in agroecosystems, and it is highly recommended to perform a sequential testing scheme of semi-field and field evaluations as the International Organisation for Biological and Integrated Control (IOBC) proposes. This method is closer to the real scenario of the agroecosystem.

CHAPTER V

MOLECULAR AND BIOCHEMICAL STUDIES OF RESISTANCE IN *CHRYSOPERLA EXTERNA* (NEUROPTERA: CHRYSOPIDAE)

V.1. Introduction

Pest control in Argentina is mainly based on the use of broad-spectrum pesticides as pyrethroids, organophosphates and carbamates (Capello & Fortunato 2008). The extensive and at certain extent irrational use of these pesticides, has led to failures due to high levels of insecticide resistance developed by pests and loss of natural enemies. Resistance to insecticides has been widely studied because of the economical losses, and the effectiveness depletion of the chemical control that this entails (Onstad 2013).

As it was mentioned before, other consequences of these uncontrolled activities are the negative effects over the populations of the natural enemies. Interestingly, it is also known that some of them can develop levels of insecticide resistance as well (Bashir & Crowder 1983; Pree *et al*. 1989; Hoy 1990; Pathan *et al.* 2010; Sayyed *et al.* 2010). One of the strategies of IPM programs is to combine chemical control with biological control in cases where biological agents alone are not able to control a pest population efficiently (Stark *et al.* 2007). Some authors have pointed out that due to biological, ecological and biochemical characteristics, phytophagous insects are faster than predators in the development of insecticide resistance (Pathan *et al*. 2008). Three reasons can be proposed to explain the causes of the lower number of reports of insecticide resistance in natural enemies (Rodrigues *et al*. 2013): 1) a pre-adaptation of herbivores compared to predators that confers them a faster and more efficient detoxification mechanisms given by evolutionary processes related to the polyphagy (feeding on more than one resource) (Croft & Morse 1979); 2) reduced accessibility of prey and hosts after pesticide applications which led to death of the predators or departure out of the crop; and 3) lack of documentation on insecticide resistance cases with natural enemies compared with pests (Tabashnik & Johnson 1999).

The terms tolerance and resistance have been used indistinctly throughout the scientific literature. Tolerance can be defined as a natural tendency of any species or even a life stage, while resistance involves the selection of specific heritable traits in a population in response to the contact with a chemical (Coles & Dryden 2014). In this sense, tolerance or resistance to pesticides in a natural enemy could be an optimal quality leading to organisms physiologically prepared to confront toxic conditions in an agroecosystem.

Two principal mechanisms are involved in the resistance development to pyrethroids: a) mutations in the target site, the *para*-type sodium channel gene, causing a change in affinity between the insecticide and its binding site that reduces sensitivity to the insecticide (knockdown resistance or *kdr*), and b) metabolic detoxification of pyrethroids before they reach their target site by detoxification enzymes (P450 mono-oxygenases, esterases and transferases) (Zhong *et al.* 2013). Since its first report in the house fly, *kdr* or *kdr-like* resistance has been documented globally in almost all agriculturally important arthropod pests and disease vectors, with more than 30 different sodium channel mutations (Rinkevich *et al.* 2013), leading to an inevitable reduction of pyrethroids effectiveness (Dong *et al.* 2014).

This chapter deals with the Neotropical generalist predator *Chrysoperla externa* Hagen (1861) that is considered an important biological control agent in South America. The massive rearing and subsequent release in the field of *C. externa* has been promoted in several countries (Carvalho *et al*. 2002; Pappas *et al*. 2011; de Fátima *et al*. 2013). Previous studies have shown some levels of tolerance against pyrethroid insecticides even in susceptible populations (Rimoldi *et al.* 2008; 2010).

The Palearctic species *Chrysoperla carnea* is a promising candidate for IPM programs worldwide (Tauber *et al*. 2000; McEwen *et al*. 2001) due to its wide prey range and geographical distribution, resistance/tolerance to pesticides, voracious larval feeding

capacity as well as commercial availability (Medina *et al.* 2003). Resistance or tolerance against pyrethroid insecticides in this species has been investigated by several authors (Ishaaya & Casida 1981; Bashir & Crowder 1983; Pree *et al*. 1989; Pathan *et al.* 2008; 2010; Sayyed *et al*. 2010) Interstingly, this tolerance was associated with a high activity of detoxification enzymes (Ishaaya & Casida 1981; Pree *et al*. 1989), but so far there are no recent updates on this mechanism in *C. carnea*.

The objective of this work was to determine if pyrethroid insecticide resistance mechanisms exist in a field-collected population of *C. externa,* compared with a susceptible population that has been reared in the laboratory for several years, and if so, which of these mechanisms are present. For this, analyses at molecular level were performed, searching the DNA sequence of the sodium channel gen. In combination, enzymatic concentration, residual analysis and susceptibility assays were performed. The hypothesis was that *C. externa* field population individuals have resistance mechanisms to cypermethrin compared with a laboratory colony.

V.2. Materials and methods

These experiments were performed in the Laboratory of Agrozoology, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University (UGent), Belgium.

V.2.1. *Chrysoperla externa* colonies

Two colonies of *C. externa* were reared in the laboratory: a laboratory colony and a field colony. The laboratory strain was reared since 2006 in the laboratory of Ecotoxicology,

Center of Parasitological Studies and Vectors (CEPAVE – National University of La Plata, Argentina) without exposure to pesticides.

The field colony (represented by the F1) was collected in vegetable crops in the Horticultural Belt of La Plata (CHP, Buenos Aires Province, Argentina) with monthly sprayings of pyrethroids (cypermethrin, deltamethrin, lambda-cyhalothrin) and neonicotinoids (imidacloprid, thiamethoxam, acetamiprid). This strain was also refreshed with new field material from the same crop.

Both colonies were transported to Belgium in little ventilated containers, mainly as pupae and eggs, due to the protective characteristics of these two stages, and the prolonged inactive time proper of them.

Once in the UGent laboratory, the two populations were maintained under controlled conditions (25 ± 2°C; 70 ± 5%; 16:8 L:D) and were placed in ventilated plastic containers (15 cm diameter, 9 cm height) covered with a fine mesh. Adults were fed on an artificial diet (Vogt *et al.* 2000). Larvae were maintained on an *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae) colony as prey, in pea plants (*Pisum sativum* L.) (Fig. V.1).

Fig. V.1. Pea aphids of *Acyrthosiphon pisum* were reared on pea plants at UGent laboratory, and used as prey for *C. externa*

V.2.2. Chemicals

The insecticide used was zeta-cypermethrin [(S)-α-cyano-3-phenoxybenzyl (1RS)-cis-trans-3-(2, 2-dichlorovinyl)-2, 2-dimethylcyclopropanecarboxylate] (Fury 100 EW, 10% zeta-cypermethrin; Belchim Crop Protection, Londerzeel, Belgium). The maximum field recommended concentration (MFRC) was evaluated and it corresponds to 6 mg a.i./L.

The insecticide synergist utilized was piperonyl butoxide (PBO), that inhibits the P450 mono-oxygenase enzyme activity, and DEF (S,S,S-tributyl phosphorotrithioate) that inhibits esterase enzymes. The insecticide:synergist ratio was 1:10, and the topical application took place 6 h after the insecticide treatment.

For the non-metabolized pyrethroids detection by gas chromatography, the insecticide cypermethrin [(*RS*)-α-cyano-3-phenoxybenzyl (1*RS*,3*RS*;1*RS*,3*SR*)-3-(2,2-dichlorovinyl)-2,2- dimethylcyclopropanecarboxylate; Glextrin 25, 25% cypermethrin, Gleba S.A.] as standard, and its MFRC corresponds to 25 mg a.i./L. This concentration was detectable by the gas chromatograph (see Section Gas chromatography).

V.2.3. Toxicity of zeta-cypermethrin in third stage larvae of *C. externa*

Less than 24h-old larvae of the third instar were exposed to zeta-cypermethrin alone, PBO+zeta-cypermethrin and DEF+zeta-cypermethrin. Three treatments were performed in each colony: 1) zeta-cypermethrin exposure, 2) PBO+zeta-cypermethrin, 3) DEF+zeta-cypermethrin. The solutions were prepared using acetone (analytical grade) as dissolvent. Larvae were treated topically with a Hamilton® micro-applicator. A droplet of 1 µl was applied to the dorsal thorax of the larvae. Three independent replicates of ten individuals by treatment were analyzed.

The recovery proportion of individuals were registered at every 3 h after treatments. The chosen criteria for considering an affected individual was the lack of movement.

V.2.4. Non-metabolized cypermethrin analysis by gas chromatography

Larvae treated with cypermethrin alone and in combination with the enzyme synergists PBO and DEF were extracted, and the amounts of parent/non-metabolized cypermethrin were analyzed by Gas Chromatography – Electron Capture Detector (GC-ECD) (Agilent 6890N). For the standard curve, six concentrations of cypermethrin were done: 0.05, 0.1, 0.5, 1, 10 and 15 ng (Figure V.2).

After 9 h of treatment, 30 mg of *C. externa* larvae were crushed with a spoon in 20 ml of hexane and then shaking at 35 kHz for 2 minutes in an ultrasonic machine (Transonic 700, Transonic Engineers Pvt. Limited, Uttar Pradesh, India). The mixture was filtered with a Whatman n°2 filter paper, evaporated and then dissolved in 2 ml of hexane. Three replicates were performed. Samples were placed in the gas chromatograph, with the following conditions:

Injection temperature: 280°C.

Column temperature: initially at 60°C heating to 20°C/min until 150°C; heating to 15°C/min until 250°C; heating to 30°C/min until 270°C; heating to 30°C/min until 280°C.

Detector temperature: 300°C.

Portable Gas: Helium, 1 ml/min constant.

Injection volume: 1 µl.

Fig. IV.2: a) Standard solutions of cypermethrin to perform the calibration and quantification line. b) Setup of evaporation to concentrate the extract.

V.2.5. Enzymes concentration

According to the results of GC-ECD, the cytochrome P450 mono-oxygenase enzyme activity was determined *in vitro* with p-nitroanisol as a general substrate (Feyereisen 1999). Larval abdomens were homogenized in potassium phosphate buffer (0.1 M, pH 7.4) centrifuged for 5 min at 1000g and its supernatant centrifuged for another 15 min at 12000 g. The resulting supernatant was used as enzyme solution. The reaction mixture consisted of 75 µl of enzyme solution in potassium phosphate buffer, 115 µl of p-nitroanisol (2 mM), and 10 µl on NADPH (0.5 mM).

The incubation was initiated with the addition of p-nitroanisol to the reaction mix, and lasted for 10 min in 27°C. The absorbance was measured at 595 nm with a spectrophotometer (Powerwave X340, BioTek Instruments, Inc., Winooski, VT, USA). Protein concentration of the resulting supernatant was determined by a Coomassie Blue (Bradford) dye reagent (Sigma) using bovine serum albumin as a standard. Total mixed-function microsomal oxidases P450 enzyme activity was expressed as mM of enzyme activity per mg of protein. Three replicates were performed.

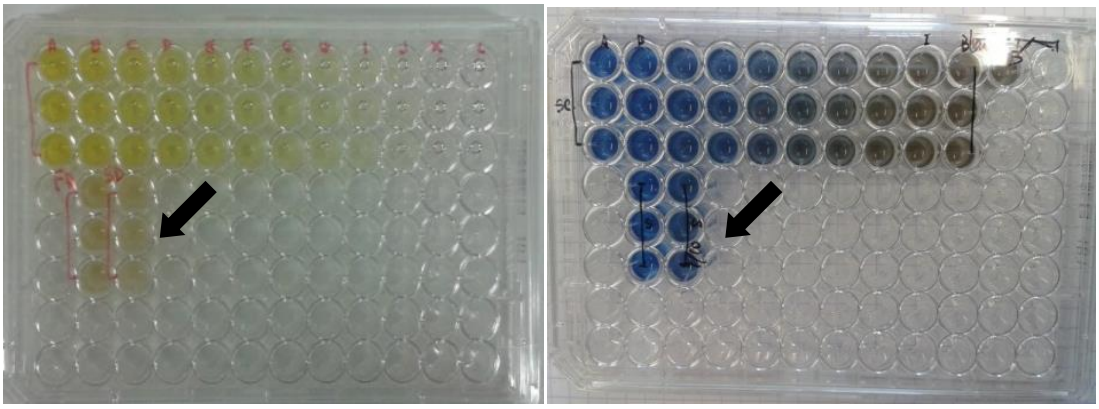


Fig. V.3: Coomassie (Bradford) method (a) Colorimetric detection of the protein concentration in the sample; (b) Colorimetric detection of the P450 mono-oxygenase enzyme activitie. From left to right, first three rows, decreasing protein concentration (a) and enzyme concentrations (b) for the calibration curves. Arrows mark the samples of proteins from field and laboratory colonies of *C. externa*.

V.2.6. cDNA partial sequence

In order to find point mutations in the sodium channel gene of *C. externa*, PCR and DNA sequencing were done. For each strain (without any treatment), total RNA was extracted from third-instar larvae using the RNeasy Mini kit (250) (Qiagen) and cDNA sequence was performed using the SuperScriptTM III Reverse Transcriptase (Invitrogen).

Degenerated primers (Table V.1) were used due to the lack of available sequence data on the sodium channel of Neuropterans. Gene sequences already known for different orders and families were aligned, with the objective to increase the range of possibilities to find the complementary DNA sequence. With the Vector NTI Software, sequences were aligned for the following species: *Anopheles gambiae* Giles (Diptera: Culicidae), *Musca domestica* L. (Diptera: Muscidae), *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), *Myzus persicae* Sulz. (Hemiptera: Aphididae) and *Bombyx mori* L. (Lepidoptera: Bombycidae). With this information, the primers were bought with LGC

Genomics GmbH (Berlin, Germany). PCR of the single-stranded cDNA was carried out with the degenerated primers and it consisted of one cycle of 95°C for 5 min and 35 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 30 s and a final extension step at 72°C for 5 min. Subsequently, the PCR product was sent for sequencing to LGC Genomics GmbH for identification.

Table V.1. Degenerated *primers* nucleotide base coding (IUPAC) for the redundant positions in the primers: R = A, G; Y = C, T; M = A, C; K = G, T; S = C, G; W = A, T; H = A, C, T; B = C, G, T; V = A, C, G; D = A, G, T; N = A, C, G, T. F: forward *primer*, R: reverse *primer*. The colored *primer* in the table corresponds to the positive one.

Primer	Sequences
F1	GGC NYT NGA YCA YCA YGA YAT G
R1	GCR AAN ARY TGN CAN CCC AT
F2	GYA THY TNA TGA TAA TGC CNA C
R2	CTC CCA GAA RTC YTG NGT C
F3	GNY TNG TNA ARG GNG CAN ARG G
R3	TGG ACG GGC TCA GAC G
F4	TGT TTT AAG GGC ACG CAA TA
R4	GCY TTS GAY TCY TCY GCY TAN GG
F5	CTG TTT TAA GGG CAC GCA ATA
R5	GCY TTS GAY TCY TCY GCY TAN GG
F6	TTA AGG GCA CGC AAT ACT CG
R6	GCY TTS GAY TCY TCY GCY TAN GG
F7	GCA CGC AAT ACT CGA AAT GTT
R7	GCY TTS GAY TCY TCY GCY TAN GG
F8	GCA CGC AAT ACT CGA AAT GTT
R8	CAT DGG VGT VAC BGC YTT SGA
F9	GGA CGA TAG CTA CTG TTT TA
R9	TGC AWY AAY TCV GTY AAY TTC CA
F10	GGA CGA TAG CTA CTG TTT TA
R10	TGC ATW CCC ATB ACB GCA AA
F11	GGA CGA TAG CTA CTG TTT TA
R11	CAY TCC CAR ACY CAY AA
F12	GGA CGA TAG CTA CTG TTT TA
R12	ACC ATD ACY TCT RMC ATY TC
F13	CGA TAG CTA CTG TTT TAA GG
R13	ACD CGY AAR ACH ATC ATY AA
F14	CGA TAG CTA CTG TTT TAA GG
R14	TGC AWY AAY TCV GTY AAY TTC CA
F15	CGA TAG CTA CTG TTT TAA GG
R15	TGC ATW CCC ATB ACB GCA AA
F16	CGA TAG CTA CTG TTT TAA GG
R16	CAY TCC CAR ACY CAY AA

F17	CGA TAG CTA CTG TTT TAA GG
R17	ACC ATD ACY TCT RMC ATY TC
F18	GCA ATA CTC GAA ATG TTC T
R18	ACC ATD ACY TCT RMC ATY TC
F19	GGA CGA TAG CTA CTG TTT T
R19	TGC AWY AAY TCV GTY AAY TTC CA
F20	GGA CGA TAG CTA CTG TTT T
R20	TGC ATW CCC ATB ACB GCA AA
F21	GGA CGA TAG CTA CTG TTT T
R21	CAY TCC CAR ACY CAY AA
F22	GGA CGA TAG CTA CTG TTT T
R22	ACC ATD ACY TCT RMC ATY TC
F23	CAA TAC TCG AAA TGT TCT TA
R23	TGC ATW CCC ATB ACB GCA AA
F24	CAA TAC TCG AAA TGT TCT TA
R24	CAY TCC CAR ACY CAY AA
F25	CAA TAC TCG AAA TGT TCT TA
R25	ACC ATD ACY TCT RMC ATY TC

V.2.7. Statistics

For the toxicity assays a two-way ANOVA was used. Effects of the different treatments (factor *treatment*) and of the populations (factor *strain*) were tested and potential interactions between these two factors on the number of recovered individuals were analyzed. For GC and enzyme activity data, a one-way ANOVA was used. If data did not accomplish the assumptions of the ANOVA, the non-parametric Kruskal-Wallis was performed. Subsequently, *Fisher's* Least Significant Differences (LSD) and Dunn post hoc tests were carried out. $P < 0.05$ was considered significant. XLSTATSTART.exe (2014) was used.

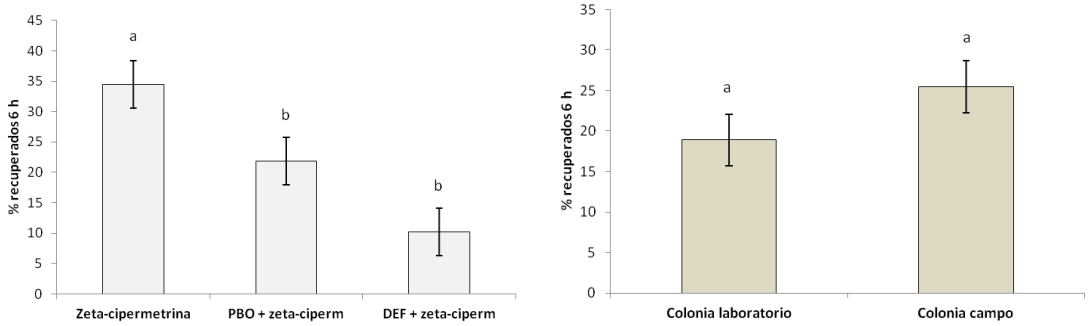
V.3. Results

V.3.1. Toxicity of zeta-cypermethrin in third stage larvae of *C. externa*

The insect susceptibility analysis demonstrated that after 3 h of treatment, 100% of

the individuals were affected. At 6 and 9 h after treatment, the factors colony and treatment acted independently on the toxicity (Fig. V.5). At 6 h, significant differences between treatments, but not between colonies, were registered (Treatment x colony interactions*: F = 0.48; df = 2; P = 0.62; treatment Factor: F = 9.67; df = 2; P = 0.0032; colony Factor: F = 2.1; df= 1; P = 0.17*). The number of recovered individuals with the single treatment with zeta-cypermethrin was higher than in the combined treatments with zeta-cypermethrin+enzyme synergist (PBO and DEF). At 9 h, no differences were registered (Treatment x colony interactions*: F = 1.73; df = 2; P = 0.21; treatment Factor: F = 3.61; df = 2; P = 0.059; colony factor: F = 3.61; df = 1; P = 0.08*).

a)



b)

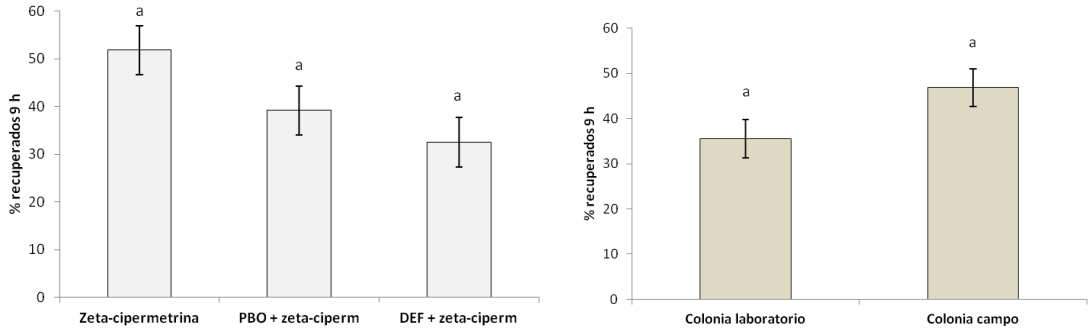


Fig. V.5. Percentage of recovered individuals with the different treatments in the laboratory and field colony. (a) 6 h post-treatment, (b) 9 h post-treatment. Data are mean ± SE. Different letters denote significant differences. (*Fisher's LSD, $\alpha = 0.05$*)

V.3.2. Non-metabolized cypermethrin analysis by gas chromatography

With the treatment of zeta-cypermethrin+PBO, laboratory and field colonies showed higher concentrations of cypermethrin residues than individuals treated with zeta-cypermethrin alone and zeta-cypermethrin+DEF (Fig. V.6). Besides, within the PBO treatment, the concentration was higher in the field laboratory colony (Treatments over laboratory colony: *F = 101.07; df = 2,6; P < 0.001.* Treatments over field colony: *F = 245.93; df = 2,6; P < 0.001*. Cypermethrin over colonies: *F = 6.14; df = 1,4; P = 0.068.* PBO+cypermethrin over colonies: *F = 437.29; df = 1,4; P < 0.001.* DEF + cypermethrin over colonies: *F = 5.96; df = 1,4; P = 0.071*).

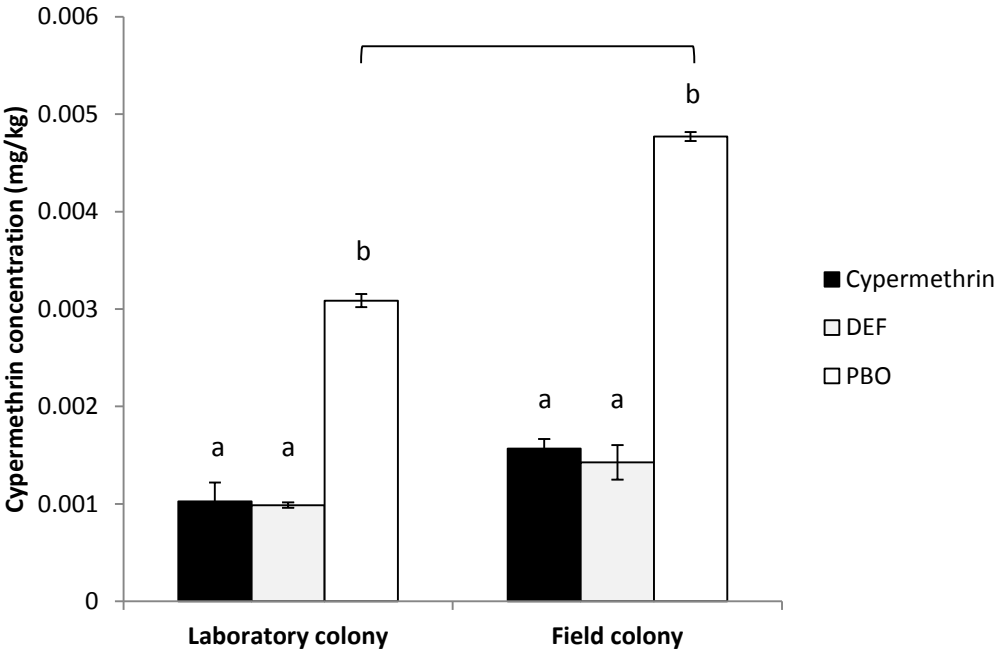


Fig. V.6. Gas chromatography data of cypermethrin residues in *C. externa* larvae. Data are mean ± SE. Different letters denote differences between treatments and the bracket denotes differences between colonies (*Fisher's LSD, $\alpha = 0.05$*)

V.3.3. Enzymes concentration

As shown in Figure V.7, the activity of the complex of P450 mono-oxygenase enzymes was not different between both susceptible and field strains of *C. externa* (*H = 0.6; df = 1; P = 0.4385*). The enzyme activity was about 1.5 mM per gram of protein.

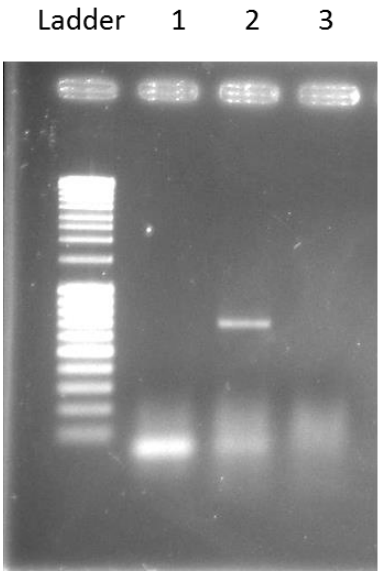
Fig. V.7. P450 mono-oxygenase enzyme activity in the laboratory and field colonies of *C. externa*. Data are mean ± SE. Same letters denote no significant differences (*Dunn, $\alpha = 0.05$*).

V.3.4. cDNA partial sequence

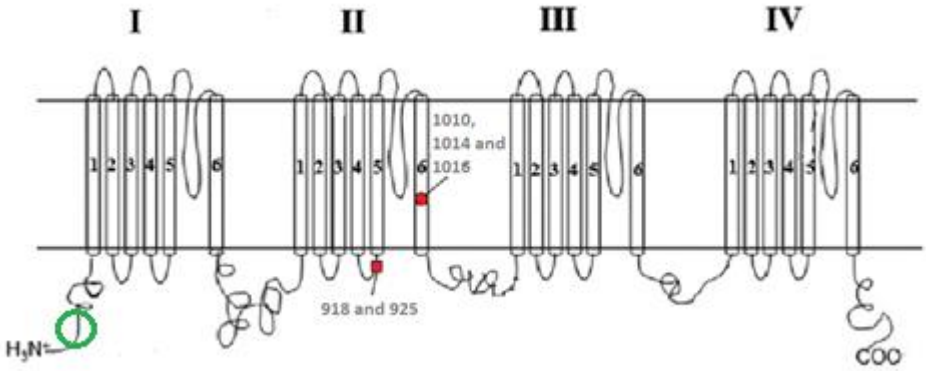
Since no sequence data for this gene in *C. externa* was available before this project, degenerated primers were designed to try to amplify and sequence the gene. Thirty degenerated primers, located in different domains of the voltage-dependent sodium channel gene, were designed and used in PCR. However, we only managed to successfully amplify a fragment with one pair of the primers, which is highlighted in Table V.1. This amplicon, around 670bp long was put on gel (Fig V.8a, lane 2). The sequencing itself however only delivered a 66bp-fragment. Fig. V.8d shows the sequence alignment of this fragment with the homologous gene in four other insect species (*An. gambiae*, *L. decemlineata*, *M. domestica*, *B. mori* and *M. persicae*). The position of the degenerate

primer is marked in red and the fragment of *C. externa* is marked in green. Sequence identities and similarities for the *C. externa* sequence and the homologous sequences were calculated using Ident and Sim (http://www.bioinformatics.org/sms2/ident_sim.html), and ranged from 75.8% to 84.9%, depending on the species (Table V.2). The sequences between laboratory and field colonies were not possible to compare, first of all because only one of the strains showed a positive result, and also because the sequence obtained was too short.

a)



b)



c) 3'ACATTCGGATGGGCATTCTTGTCTGCCTTTCGTCTAATGACTCAAGATTATTGGGAGAAT**5'**

d)

```
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            490        500        510        520        530        540
Myzus persicae      GTAACAATCA TCACTACTAT ACTTACAAAC TGTGTGTTCA TGATAATGCC CCCAACTCCG
Musca domestica     TTATTCATTA TCACCACTAT TCTAACTAAT TGTATTTTAA TGATAATGCC GACAACGCCC
Anopheles gambiae   CTTTTTATAA TAACGACCAT TCTTGTTAAT TGTATATTGA TGATTATGCC TACCACGCCG
Bombyx mori         CTGTTCATTA TTACGACAAT CTTAGTGAAT TGCATACTTA TGATAATGCC TACAACGCCA
Leptinotarsa decemlineata ---------- ---------- ---------- ---------- ---------- ----------
Chrysoperla externa ---------- ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            550        560        570        580        590        600
Myzus persicae      ACTATTGAAG CGTCTGAAGT AATATTTACC GGCATCTACA CATTCGAATC GGCTGTGAAA
Musca domestica     ACGGTCGAAT CCACAGAGGT GATATTCACC GGAATCTACA CATTTGAATC AGCTGTTAAA
Anopheles gambiae   ACAGTCGAAT CTACCGAGGT GATATTCACC GGCATCTACA CGTTCGAATC AGCTGTAAAA
Bombyx mori         ACAGTTGAAA GTACTGAAGT TATCTTTACC GGGATCTACA CGTTTGAATC AGCGGTGAAA
Leptinotarsa decemlineata ---------- ---------- ---------- ---------- ---------- ----------
Chrysoperla externa ---------- ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            610        620        630        640        650        660
Myzus persicae      GTAATGGCCC GAGGTTTCAT ATTAGAACAC TTCACCTATC TTAGAGATGC ATGGAATTGG
Musca domestica     GTGATGGCAC GAGGTTTCAT TTTATGCCCG TTTACGTATC TTAGAGATGC ATGGAATTGG
Anopheles gambiae   GTGATGGCGC GAGGTTTCAT ATTACAACCG TTTACTTATC TTAGAGATGC ATGGAATTGG
Bombyx mori         GTAATGGCCA GGGGTTTTAT ACTACAGCCA TTCACATACC TTAGAGATGC ATGGAATTGG
Leptinotarsa decemlineata ---------- ---------- ---------- ---------- ---------- ----------
Chrysoperla externa ---------- ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            670        680        690        700        710        720
Myzus persicae      CTAGACTTCA TTGTTATTGC ATTAGCTTAC GTTACTATGG GTATAGAACT TGGAAATTTA
Musca domestica     CTGGACTTCG TAGTAATAGC TTTAGCTTAT GTGACCATGG GCATAGATTT AGGTAATCTC
Anopheles gambiae   TTGGACTTCG TAGTAATAGC ATTAGCATAT GTAACTATGG GTATAGATTT GGGTAATCTT
Bombyx mori         CTTGACTTCG TAGTTATAGC TTTAGCTTAT GTGACGATGG GCATAGATCT CGGAAACCTA
Leptinotarsa decemlineata ---------- ---------- ---------- ---------- ---------- ----------
Chrysoperla externa ---------- ---------- ---------- ---------- ---------- ---AAATCTA

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            730        740        750        760        770        780
Myzus persicae      GCGGTTCTTC GAACATTTCG AGTACTGCGA GCGCTCAAAA CTGTAGCCAT TGTGCCTGGA
Musca domestica     GCAGCTTTGA GAACATTTAG GGTACTGCGA GCTCTGAAAA CCGTAGCCAT TGTGCCAGGT
Anopheles gambiae   GCTGCGTTGA GAACATTCAG GGTATTACGA GCTCTTAAAA CGGTAGCCAT CGTTCCAGGC
Bombyx mori         GCCGCTCTGA GAACGTTCAG GGTACTACGA GCGTTGAAGA CTGTGGCCAT AGTACCGGGC
Leptinotarsa decemlineata ---------- ---------- ---------- ---------- ---------- ----------
Chrysoperla externa GCCGCCTTAA GAACATTTCG AGTATTGCGT GCCCTTAAAA CAGTAGCTAT CGTCCCAGG-

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            790        800        810        820        830        840
Myzus persicae      TTAAAGACTA TCGTTGGAGC TGTGATAGAA TCCGTGAAAA ACCTCAGGGA TGTCATAATA
Musca domestica     CTAAAAACCA TTGTCGGTGC TGTCATTGAA TCTGTAAAAA ATCTACGCGA TGTGATAATT
Anopheles gambiae   TTAAAAACCA TCGTCGGAGC CGTTATAGAA TCCGTAAAGA ATCTCAGAGA TGTGATAATT
Bombyx mori         TTGAAGACGA TCGTCGGTGC TGTTATAGAA TCCGTGAAAA ATCTCCGTGA TGTAATAATT
Leptinotarsa decemlineata ---------- ----TGGAGC TGTAATAGAA TCAGTAAAAA ATCTCCGAGA TGTGATAATT
Chrysoperla externa ---------- ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            850        860        870        880        890        900
Myzus persicae      TTAACAATAT TTTCACTATC TGTGTTCGCA TTACTGGGAT TACAAATTTA TATGGGTGTG
Musca domestica     TTGACAATGT TTTCCCTGTC GGTGTTCGCG CTGATGGGCC TACAAATCTA TATGGGTGTT
Anopheles gambiae   TTAACAATGT TTTCGTTATC CGTGTTTGCT TTGATGGGTC TACAAATCTA CATGGGAGTA
Bombyx mori         TTAACGATGT TTTCGCTGTC CGTATTCGCG TTGATGGGCC TACAGATTTA CATGGGAGTT
Leptinotarsa decemlineata TTAACAATGT TTTCTCTTTC CGTTTTTGCG CTAATGGGTT TACAAATTTA TATGGGTGTC
Chrysoperla externa ---------- ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            910        920        930        940        950        960
Myzus persicae      CTA-ACACAA AAATGTATAA AATATTTTCC CCTTGACGGT TCAGCCGGAA ATTTAACCAA
Musca domestica     CTA-ACACAA AAGTGCATTA AACGATTCCC CCTGGACGGC AGTTGGGGCA ATCTGACCGA
Anopheles gambiae   CTA-ACACAA AAGTGCATAA AAGAGTTCCC ATTGGATGGT TCCTGGGGTA ATCTAACCGA
Bombyx mori         TTG-ACGCAG AAGTGTGTCA AAGTGTTCCC GGAGGACGGC TCTTGGGGTA ACCTGACCGA
Leptinotarsa decemlineata CTC-ACACAA AAATGTATTA AAAACTTTCC ATTAGACGGC TCCTGGGGAA ATCTCACGGA
Chrysoperla externa ---------- ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            970        980        990       1000       1010       1020
Myzus persicae      TGAAAATTGG GTTGCGTTCA TGTCGAATAA ATCAAATTGG CAGCCCGGTG ATGAGGAACC
Musca domestica     TGAAAACTGG TTTCTACACA ATAGCAACAG TTCCAATTGG TTTACGG--- AGAACGATGG
Anopheles gambiae   CGAAAGCTGG GAGCTGTTCA ACAGCAATGA CACAAATTGG TTCT------ ATTCCGAGAG
Bombyx mori         CGAGAACTGG GAGAGGTTTT GTCAGAACGA AACTAACTGG TA-------- -TGGTGAAGG
Leptinotarsa decemlineata CGAAAACTGG GAAAGATTCA CAAGTAATGA AACCAACTGG TACG------ TGGATTCCAA
Chrysoperla externa ---------- ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            1030       1040       1050       1060       1070       1080
Myzus persicae      AGAAGATTAT CCATTATGCG GAAACGGTAC AGGCGCTGGT CAATGCAAAG AAGGTTATAT
Musca domestica     CGAGTCATAT CCGGTGTGCG GGAATGTATC CGGTGCGGGA CAATGCGGCG AAGATTACGT
Anopheles gambiae   TGGCGACATT CCTCTTTGTG GAAACTCATC TGGAGCTGGA CAATGTGATG AAGGCTACAT
Bombyx mori         GGGTGATTAT CCTTTATGCG GCAATTCATC AGGGGCAGGA CAATGCGAAC CGGGCTACGT
Leptinotarsa decemlineata CGGGGACAAA CCACTTTGTG GAAATTCCTC TGGGGCAGGT CAATGCAAAG CCGGTTACAC
Chrysoperla externa ---------- ---------- ---------- ---------- ---------- ----------
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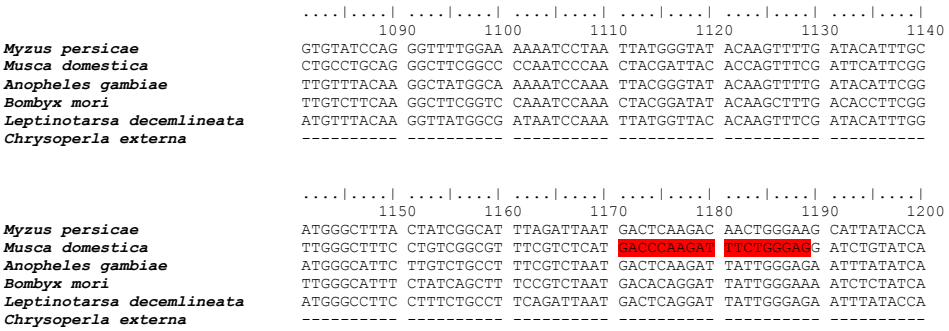

Fig. V.8. a) Agarose gel with EtBr staining, containing the PCR products; the first lane corresponds to the Mass Ruler™ DNA Ladder Mix. Lanes 1-3 corresponds to the PCR products obtained using primers F1R1, F2R2 and F3R3, respectively. In lane 2, a clear single band of around 670bp could be detected (Table V.1). b) Structure of the voltage-gated sodium channel showing the domains (I – IV), each one having 6 transmembrane segments. Red dots are the main pyrethroid resistance mutations. The green circle marks the region in the protein, which corresponds to the obtained cDNA sequence. c) cDNA sequence d) Nucleotide sequence alignment (which corresponds only to domain I of the protein; the rest of the sequence could not be aligned) between the Na-channel sequences for *C. externa*, *An. gambiae*, *L. decemlineata*, *M. domestica*, *B. mori* and *M. persicae*. The positions of the degenerate primers used for amplification and sequencing are highlighted in red. The partial fragment obtained for *C. externa* is highlighted in green.

Table V.2. Sequence identity and similarity results on nucleotide level using Ident and Sim. Note: no comparison could be made between *C. externa* and *L. decemlineata* since only a partial sequence is available for the latter.

	Alignment length	Identical residues	Similar residues	Percent identity	Percent similarity
Chrysoperla* vs *Anopheles	66	52	4	78.79	84.85
Chrysoperla* vs *Leptinotarsa	-	-	-	-	-
Chrysoperla* vs *Musca	66	51	2	77.27	80.30
Chrysoperla* vs *Bombyx	66	45	7	68.18	78.79

Chrysoperla* vs *Myzus	66	50	0	75.76	75.76

V.4. Discussion

Resistance or tolerance against pesticides in beneficial arthropods has been extensively documented, and the release of these organisms could be implemented in IPM programs. An ideal biological control agent would be one that is tolerant to synthetic insecticides (El-Wakeil *et al.* 2013) since under some circumstances pesticide spraying cannot be avoided in agroecosystems. Inundative releases of *C. carnea* were effective in controlling populations of pest complexes in several crops (Simmons & Abd-Rabou 2011), but the use of this species in IPM has increased during the recent decades because of the advantage of relatively broad tolerance to many insecticides, particularly during the larval and cocoon stages (Ishaaya & Casida 1981; Grafton-Cardwell & Hoy 1985; Pree *et al*. 1989; Medina *et al*. 2001). All stages of *C. carnea* are highly tolerant of many synthetic pyrethroids, botanicals, microbial insecticides (*Bacillus thuringiensis* and nuclear polyhedrosis viruses), insect growth regulators, fungicides, herbicides, acaricides (Grafton-Cardwell & Hoy 1985). Regarding pyrethroid isecticides, Grafton-Cardwell & Hoy (1985) suggested that the high tolerance of *C. carnea* is probably due to its natural tolerance.

The Neotropic *C. externa* has been considered a very important natural enemy in South America for its generalist foraging behavior and strong adaptability to different ecosystems (de Fátima *et al.* 2013; Moura *et al*. 2011). Its presence in treated fields could be a result of tolerance or resistance processes to certain agrochemicals. For instance, Moura *et al*. (2011) found a low mortality rate of *C. externa* with sulfur, abamectin and trichlorfon, and

they concluded that these could be related to a high tolerance.

Regarding the toxicity results obtained in this study, all larvae were knocked-out after 3 h of treatment. Laboratory and field colonies did not show differences when exposed to cypermethrin. In both groups, individuals treated with zeta-cypermethrin alone recovered much faster than individuals treated with cypermethrin+enzyme synergist (PBO and DEF) at 6 h after treatment. At 9 h after treatment, the effect of the enzyme synergist was lost compared to the zeta-cypermethrin treatment.

The results as obtained in this chapter suggest that P450 mono-oxygenase enzymes and esterases can be involved in the metabolic detoxification of zeta-cypermethrin in *C. externa*. Other authors have reported a higher toxicity to pyrethroid insecticides when PBO and DEF were applied as pre-treatments (Alzogaray & Zerba 1997; Xi *et al.* 2015). Our results are consistent with Sayyed *et al.* (2010) who concluded that deltamethrin resistance in *C. carnea* from Pakistan was associated with higher P450 enzyme activities and possibly also with esterase enzyme activities. It is also important to underline that independent of the colony origin (laboratory and field strains), individuals had a recovery of 50% at 9 h after treatment. P450 mono-oxygenase enzymes form a very important group which, among other functions, metabolize foreign chemicals. In insects, microsomal and mitochondrial P450s are present and play a significant role in the development of resistance against insecticides (Feyereisen 1999). This resistance may be due to an upregulation of P450 enzymes or the presence of point mutations in target molecules (Wilson 2001). The use of the synergist PBO to inhibit these enzymes, has been an efficient strategy for the control of resistant pest insects (Burgess 2004), but research on the effect on natural enemies is very sparse.

The GC-ECD assays in this chapter confirm that P450 mono-oxygenase enzymes are

involved in the detoxification of cypermethrin due to the significantly higher amounts of the pesticide in samples from individuals treated with PBO (the enzyme syngerist against P450 enzymes) compared with those treated with cypermethrin alone and cypermethrin+DEF. With the cypermethrin+PBO treatment, the amount of residues in the field strain was significantly higher than in the susceptible strain. Similar results were obtained in Guerrero *et al.* (1997) where PBO+pyrethroid assays demonstrated that a resistant colony of *Haematobia irritans* had a higher synergism than a susceptible colony. This could be due to a lower contribution by P450 enzymes in the detoxification in the susceptible colony than in the resistant one.

It is remarkable the difference between the results of toxicity assays and GC-ECD data with zeta-cypermethrin and cypermethrin, regarding DEF treatment. Although belonging to the same pyrethroid group (Type II, with a cyano group in the molecule) and formed by the same molecule, these results denoted the existence of a distinctive detoxification process according to the type of cypermethrin: while zeta-cypermethrin+DEF had a minor percentage of recovered individuals compared to zeta-cypermethrin alone and zeta-cypermethrin+PBO, the residual amounts of cypermethrin were the same with the pyrethroids alone and with cypermethrin+DEF. The amounts of the insecticide with cypermethrin+PBO were two to four fold higher compared to the other treatments. This could indicate that esterases are not involved in cypermethrin detoxification, but they are involved in zeta-cypermethrin detoxification.

The P450 enzyme activities were measured and they did not show a difference between the *C. externa* strains tested. This method of residue analysis of pesticides by GC-ECD, as done in this study, was used for the first time in predators. There are some studies done in honeybees where different determinations of pesticide residues methods are

presented (Rossi *et al.* 2001; Morzycka 2002; Walorczyk & Gnusowski 2009). The present study points out the importance of including this kind of assays in natural enemies, in addition to the lethal and sub-lethal effects evaluation, in order to increase knowledge on the impact of pesticides in beneficial insects in an environmental framework.

On the other hand, the cDNA synthesis and sequencing of the sodium channel of *C. externa* should be of a great importance. Such sequencing has been done before for various arthropods of economical and health importance (i.e. *M. domestica*, mosquitoes *An. gambiae*, *A. aegypti*, *Culex quinquefasciatus* Say, the potato pest coleopteran *L. decemlineata*, the whitefly *Bemisia tabaci* Gennadius, etc.; NCBI, www.ncbi.nlm.nih.gov) that had developed high levels of pyrethroid resistance with mutations in that gene. The voltage-dependent sodium channels (transmembrane proteins) are found in neurons, and they remained the polarized membrane until an action potential reaches, which is the time that the channels open and an influx of Na^+ ions enters the neuron. This is causing the membrane "depolarization" and the subsequent action potential. Pyrethroid insecticides act at this level, joining the transmembrane protein and so they cause that the neuron remains activated with the channels open (Dong *et al.* 2014). Obtaining the DNA sequence of the gene encoding this protein in *C. externa* would be not only be important to increase the knowledge of this species at a molecular level, but also for the general knowledge on the mechanism of insecticides whose target site is the sodium channel. So far, numerous studies have shown that mutations in this gene occur mainly in domain II and III of the protein (the most common are called L1014F, L1014H and M918T + L1014F, with the letters corresponding to the name of the amino acid replaced in the mutation, and the numbers to the amino acid position) (Franck *et al.* 2012;. Rinkevich *et al.* 2013;. Xu *et al.* 2012;. Dong *et al.* 2014. Singh *et al.* 2015). It is important to include this type of analysis in natural enemies

in addition to the evaluation on the lethal and sublethal effects of pesticides, in order to increase our knowledge about the mechanisms of resistance/tolerance against pesticides in beneficial insects in risk assessments programs. In this chapter, a complete DNA sequencing was unfortunately not achieved. With most of the degenerate primers, we did not succeed in amplifying a clear single band. While one degenerate primer pair did amplify a clear fragment, only a 66bp fragment of this amplicon was obtained from the sequencing. This could possibly be due to the purity and quality of the sample that was sent for sequencing, due to non-specific binding and amplification by one or both of the primers making it impossible to get, with any certainty, a correct sequence over most of the fragment length. In future studies it should be ambitioned that a complete neuronal genome should be obtained.

Based on the results of this chapter, the Neotropical lacewing *C. externa* keeps the potential to survive to pesticides, in this case the pyrethroid zeta-cypermethrin, even after six years of rearing in the laboratory without exposure to pesticides. This could demonstrate that this species has a natural tolerance against cypermethrin. The P450 enzyme activity measurements showed an important detoxification role on both strains and this was in agreement with the GC-ECD results. Taking into account that in a mass rearing program the individuals are not exposed to pesticides during their breeding and that all agricultural fields are (or at least were) exposed to chemicals, this species is a promising natural enemy and could be useful in an IPM program in the field to control important pests.

CHAPTER VI

COMPARATIVE STUDIES OF TOXICITY OF PESTICIDES BETWEEN LARVAE AND PUPAE OF *CHRYSOPERLA ASORALIS* AND *C. EXTERNA* (NEUROPTERA: CHRYSOPIDAE)

VI.1. Introduction

Many studies in literature evaluate sub-lethal and lethal effects of insecticides on natural enemies. These studies are of great importance because benefical insects are also affected by chemical control. In the Horticultural Belt of La Plata, conventional insecticides (organoclorates, organophosphates, carbamates and pyrethroids) (CASAFE 2015) remain the most utilized, although in recent years there has been an increase in the use of biorational insecticides in the region.

The Neuropteran *C. externa* is an important biological control agent, whose larvae are predators that feed on pests of economical importance (Soto & Iannacone 2008; Bastidas *et al*. 2010). Previous toxicity studies have demonstrated a high pyrethroid tolerance (Rimoldi *et al*. 2008; 2012), and see also Results presented in previous Chapters of this thesis work, just as in the Palearctic species *C. carnea* (Pree *et al*. 1989; Hoy 1990; Pathan *et al.* 2008; 2010; Sayyed *et al*. 2010). Studies done to elucidate the impact of neonicotinoids on different stages of *C. externa,* have shown a high susceptibility of the species to this insecticide (Bueno & Freitas 2006; Godoy *et al*. 2010). Regarding pyriproxyfen, studies demonstrated that it is not toxic to this species (Velloso *et al*. 1997; de Fátima *et al*. 2013).

The presence of the chrysopid *C. asoralis* has been recorded in the Horticultural Belt of La Plata (Chapter III) as well as the Neotropical and widely distributed species *C. externa. C. asoralis* has been recorded in Cuyo, Northwest and Northeast regions of Argentina and now also in Buenos Aires province (González Olazo & Heredia 2007; González Olazo *et al.* 2009; González *et al.* 2011) in association with economic important crops, such as fruit trees and olives (González *et al*. 2011). Pesticide applications are carried out according to periodical monitoring and economic injury

level establishment for *Myzus persicae* and *B. tabaci,* have demonstrated the presence of this species for the first time in the region, but it has not been observed in other survey points where conventional strategies are carried out.

The objective of this chapter was to determine if *C. asoralis* is more susceptible to insecticides than *C. externa,* since the first species is present in crops with a low frequency of insecticide applications. To cope with this objective, laboratory assays included three pesticides that are commonly used in the study region: the pyrethroid cypermethrin, the neonicotinoid acetamiprid and the insect growth regulator pyriproxyfen. We tested here in this study the effects of the applications on two developmental stages: the larval and pupal stage.

VI.2. Materials and methods

VI.2.1. Insects

Laboratory colonies of both *C. externa* and *C. asoralis* were build-up from material collected in the field (Chapter III) and maintained in the same conditions as described in previous Chapters. Cohorts of less than 24 h old were selected for experiments. The two colonies were maintained under controlled conditions (25 ± 1°C, 70 ± 5% HR and 16:8 h L:D) and were placed in ventilated plastic containers (15 cm diameter, 9 cm height) covered with a fine mesh. Larvae were fed with a *R. padi* (Hemiptera: Aphididae) colony as prey. Adults were fed on an artificial diet (Vogt *et al*. 2000).

VI.2.2. Insecticides, preparation of solutions

The following insecticides were used at their respective maximum field recommended concentration (MFRC) from the commercial formulations: Glextrin® (25% w/v cypermethrin, Gleba S.A.), Mospilan® (20% w/w acetamiprid, Summit-Agro S.A.) and Epingle® (10% w/v pyriproxyfen, Summit-Agro S.A.) (CASAFE 2015) (Table 1).

Table 1: insecticides used and their MFRC:

Active ingredient	Commercial name	Purity (w/v)	MFRC (mg a.i./L)	Company
Cypermethrin	Glextrin 25®	25%	25	GLEBA S.A.
Acetamiprid	Mospilan®	20%	200	SUMMIT AGRO S.A.
Pyriproxyfen	Epingle®	10%	75	SUMMIT AGRO S.A.

VI.2.3. Toxicity on the third instar larvae

Third instar larvae (≤ 24 h old) of *C. externa* and *C. asoralis* were treated. The mode of application was topical, using a Hamilton® micro-applicator. A droplet of 1 µl of the working solution was applied on the dorsal abdomen of each larva. The control was prepared with acetone analytical grade. The experimental unit consisted of 1 individual larva per chrysopid species, treated with the pesticide compound or acetone. Thirty repetitions by treatment were analyzed. Treated larvae were placed in little capsules of 1 cm diameter per 2 cm height.

VI.2.4. Toxicity on pupal stage

Pupae of both species were also topically treated as described for the experiment with larvae. Since pupae developed inside a silky cocoon (that encloses it), treatments were done after 72 h from pupal formation to ensure that all individuals had completed that developmental stage. Thirty repetitions by treatment were analyzed.

The susceptibility of both species were compared by measuring the following biological parameters: 1) mortality in the larval and pupal stage, 2) developmental time from larva to pupa and 3) pupa to adult; 4) pre-oviposition period, 5) fecundity and fertility, in the case individuals reached the adult stage.

To estimate pre-oviposition, fecundity and fertility, for those treatments where individuals could complete their life cycle, the adult genre was determined and then paired up, to register those reproductive parameters, during the first five days of oviposition. Five couples (repetitions) by treatment were analyzed. Each couple was placed in a container of 4 cm diameter and 6 cm height, and artificial diet and water were supplied. A black cardboard was provided inside the containers to facilitate the extraction and counting of the laid eggs. These cardboards with eggs were placed in Petri dishes and controlled during 10 consecutive days, to register the larval emergence.

VI.2.6. Statistics

One-way ANOVA was used. Normality and homoscedasticity assumptions were previously analyzed with Shapiro-Wilk and Levene tests, respectively. If data did not

accomplish the assumptions of the ANOVA, non-parametric Kruskal-Wallis was performed. Subsequently, LSD and Dunn post hoc tests were carried out. $P < 0.05$ was considered significant. XLSTATSTART.exe (2014) was used to perform the statistical analyses.

VI.3. Results

VI.3.1. Toxicity on the treated third instar larvae

VI.3.1.1. Mortality

Total number of dead larvae and pupae was registered at the end of every stage treated with different insecticides. Control treatments yielded 100 % of survivorship during the larval stage in either of the two species. Treatments with acetamiprid and cypermethrin produced highly significant mortalities compared with pyriproxyfen, which did not differ from the control (*C. asoralis* mortality: *H = 10.23; df = 2; P = 0.016*. *C. externa* mortality: *H = 6.49; df = 2; P = 0.08*) (Fig. 1). Comparing *C. asoralis* and *C. externa* mortalities with each insecticide, cypermethrin treatment was more lethal to pyriproxyfen than acetamiprid. Mortality of *C. asoralis* treated with cypermethrin was 90% and in *C. externa* 20% (mortality for two species with pyriproxyfen: *F = 0.8; df = 1,4; P = 0.42;* cypermethrin: *F = 39.97; df = 1,4; P = 0.003;* acetamiprid: *F = 0.02; df = 1,4; P = 0.9*).

Regarding pupal mortality of surviving individuals, the treatment with acetamiprid produced significantly higher mortalities compared with control and with the remaining treatments, in both species (pupal mortality of *C. asoralis* with different treatments: *F = 7.84; gl = 3,8; P = 0.009*. Pupal mortality of *C. externa*: *F = 6.1; df = 3,8;*

P = 0.018) (Fig. VI.2). On the other hand, and considering the effect of each insecticide within the species, acetamiprid caused higher mortality in *C. asoralis* than in *C. externa*, while there were no significant differences with pyriproxyfen (mortality with pyriproxyfen: *F = 0.03; df = 1,4; P = 0.86*. Mortality with acetamiprid: *F = 97.68; df = 1,4; P < 0.001*).

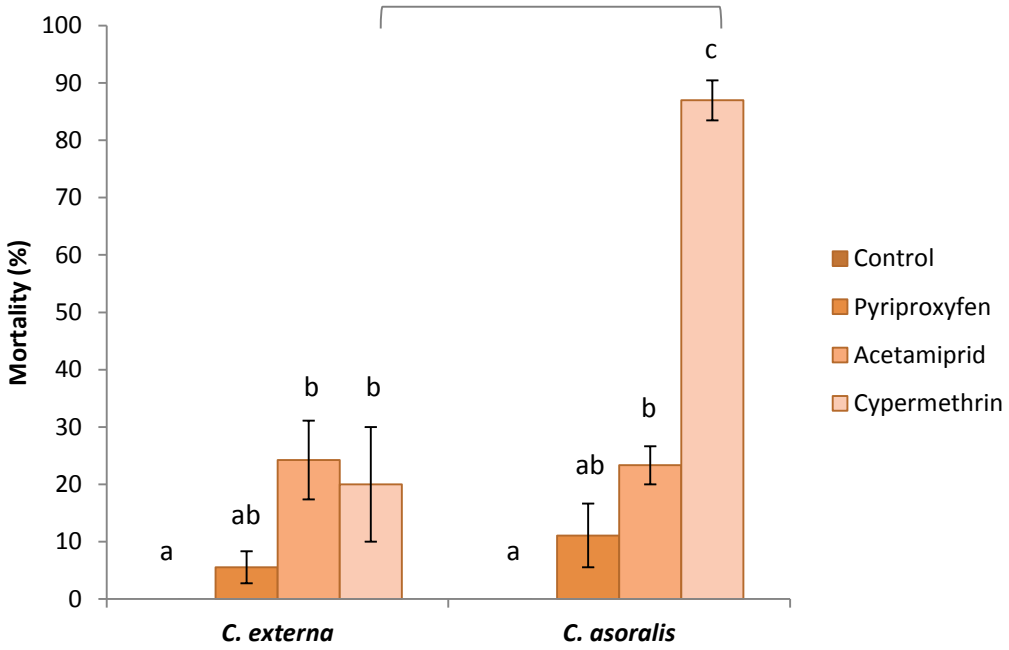

Fig. VI.1. Mortality percentage in the larval stage treated with different insecticides. Data are mean ± SE. Different letters denote significant differences between treatments within each species. Bracket marks the treatment that showed significant differences between species (*P < 0.05*)

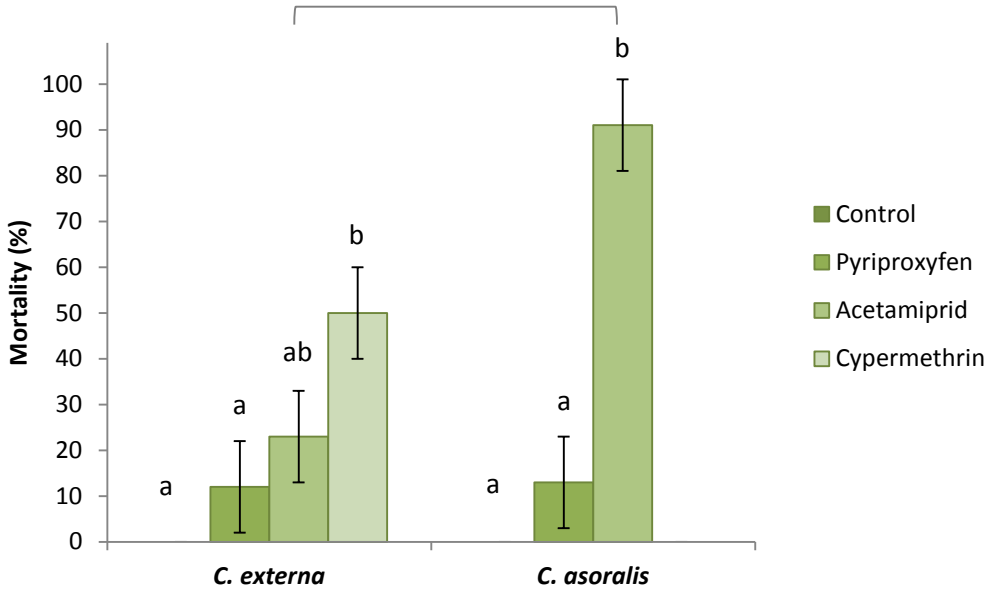

Fig. VI.2. Mortality percentage in the pupal stage treated with different insecticides. Data are mean ± SE. Different letters denote significant differences between treatments within each species. Bracket marks the treatment that showed significant differences between species (*P < 0.05*)

VI.3.1.2. Developmental time from larva to pupa

Because of the high mortality in the *C. asoralis* larval stage produced by cypermethrin, it was not possible to add this treatment in the species comparison.

C. asoralis showed a significant longer developmental period with acetamiprid, while cypermethrin and acetamiprid both elongate this period in *C. externa* when compared with the control. Larvae of *C. externa* extended their developmental time when acetamiprid was applied (larva-pupa period of *C. asoralis* with different treatments: *H = 21.63; df = 2; P < 0.001*. Larva-pupa period of *C. externa*: *H = 27.07; df = 2; P < 0.001*) (Fig. VI.3). The developmental times measured on larvae treated with pyriproxyfen and control treatments, were longer in *C. asoralis* than *C. externa,* but the opposite occurred with acetamiprid (Larva-pupa period of control: *F = 14.88; df = 1,59;*

P < 0.001. Larva-pupa period with pyriproxyfen: *F = 10.26; df = 1,60; P = 0.002*. Larva-pupa period with acetamiprid: *H = 4.46; df = 1; P = 0.03*).

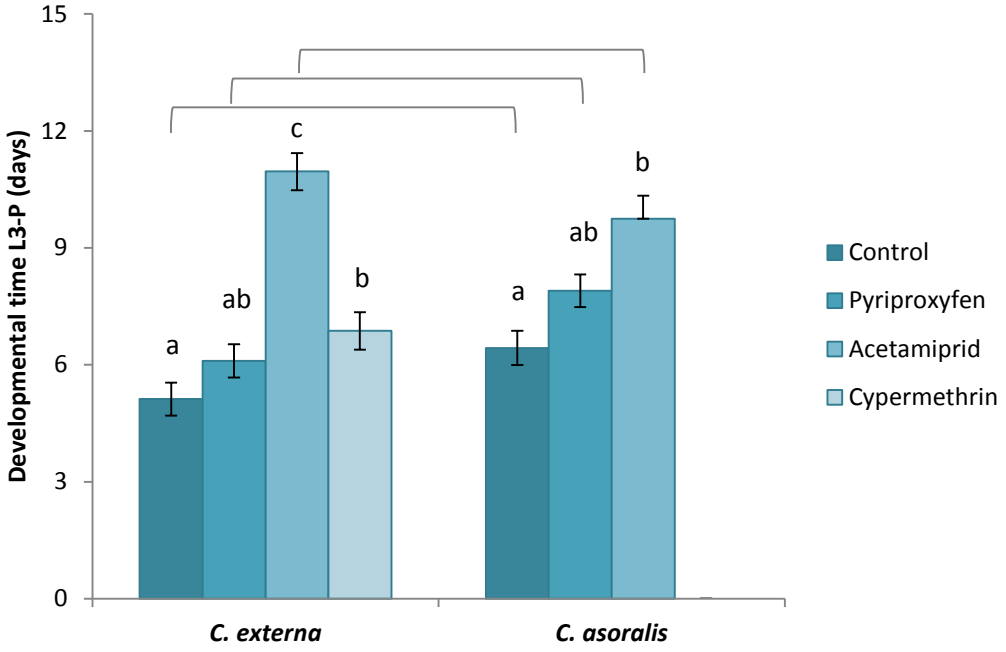

Fig. VI.3. Developmental time from larva to pupa. Data are mean ± SE. Different letters denote significant differences between treatments within each species. Brackets mark the treatment that showed significant differences between species (*P < 0.05*)

VI.3.1.3. Developmental time from pupa to adult

Chrysoperla asoralis pupae experimented high mortality and the developmental time from pupa to adult was not able to calculate, because of the lack of adults. Pyriproxyfen treatment did not prolonged the period compared with control.

On the other hand, acetamiprid treatment in *C. externa* shortened the larva-pupa period, compared with control and cypermethrin treatments, which they did not differ between them. Pyriproxyfen prolonged the larval-pupal period (Pupa-adult period of *C. asoralis*: *H = 2.43; df = 1; P = 0.11*. Pupa-adult period of *C. externa*: *H = 21.95; df = 3; P < 0.001*) (Fig. VI.4).

There were no significant differences between pyriproxyfen treatments in both species (Pupa–adult period of control: *F = 3.34; df = 1,54; P = 0.07*. Pupa-adult period with pyriproxyfen: *F = 0.14; df = 1,54; P = 0.71*).

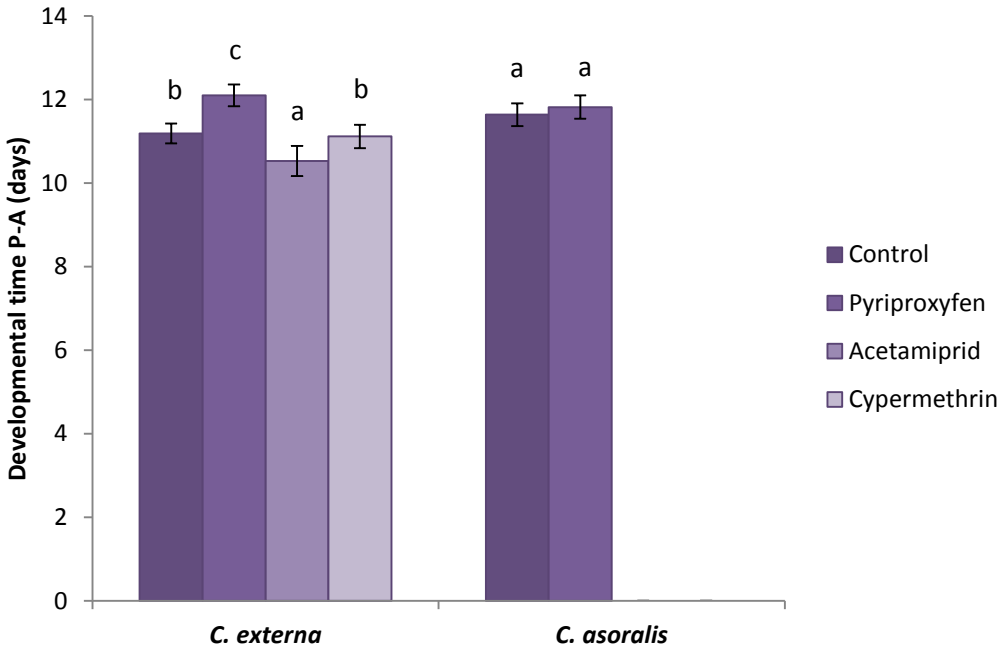

Fig. VI.4. Developmental time from pupa to adult (in days). Data are mean ± SE. Different letters denote significant differences between treatments within each species. (*P < 0.05*)

VI.3.1.4. Preoviposition period

The preoviposition period in control *C. asoralis* females was longer than in *C. externa*. When pyriproxyfen was applied the period was significantly shortened significantly only in *C. asoralis* (Pre-oviposition period of control between species: *F = 18.67; df = 1,9; P = 0.001*. Pre-oviposition period with pyriproxyfen between species: *F = 2.12; df = 1,9; P = 0.18*). *C. externa* was not affected with any of the treatments, while pyriproxyfen shortened the period in *C. asoralis* (Pre-oviposition period of *C. asoralis*: *F = 7; df = 1,10; P = 0.02*. Pre-oviposition period of *C. externa*: *F = 1.40; df = 2,13; P = 0.28*) (Fig. VI.5).

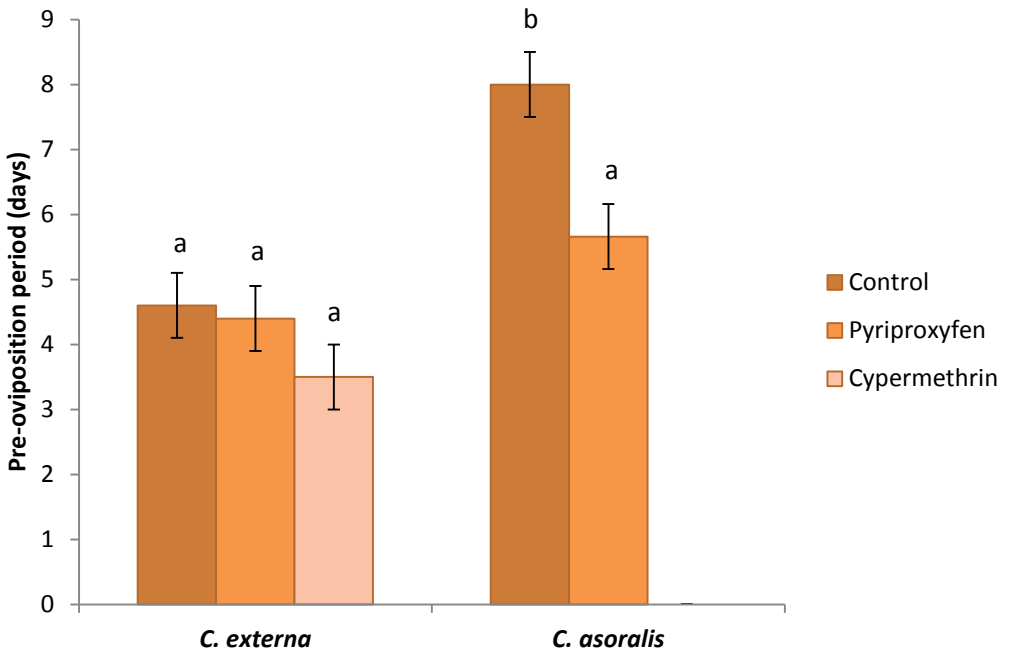

Fig. VI.5. Pre-oviposition period, time from the adult emergence until the first day of oviposition (in days). Data are mean ± SE. Different letters denote significant differences between treatments within each species (*P < 0.05*)

VI.3.1.5. Fecundity and fertility

Regarding the number of eggs laid by female and the hatched larvae, there were no significant differences between each species nor between treatments (Treatments within species: *C. asoralis* fecundity: *F = 0.96; df = 1,12; P = 0.34*. *C. externa* fecundity: *H = 4.34; df = 2; P = 0.11*. *C. asoralis* fertility: *F = 0.67; df = 1,12; P = 0.43*. *C. externa fertility*: *H = 0.45; df = 2; P = 0.79*) (Comparison between species: fecundity of control: *F = 0.22; df = 1,11; P = 0.64*. fecundity of pyriproxyfen: *F = 0.0006; df = 1,15; P = 0.98*. fertility of control: *F = 0.03; df = 1,11; P = 0.86*. fertility of pyriproxyfen: *H = 0.27; df = 1; P = 0.6*) (Fig. VI.6 and VI.7).

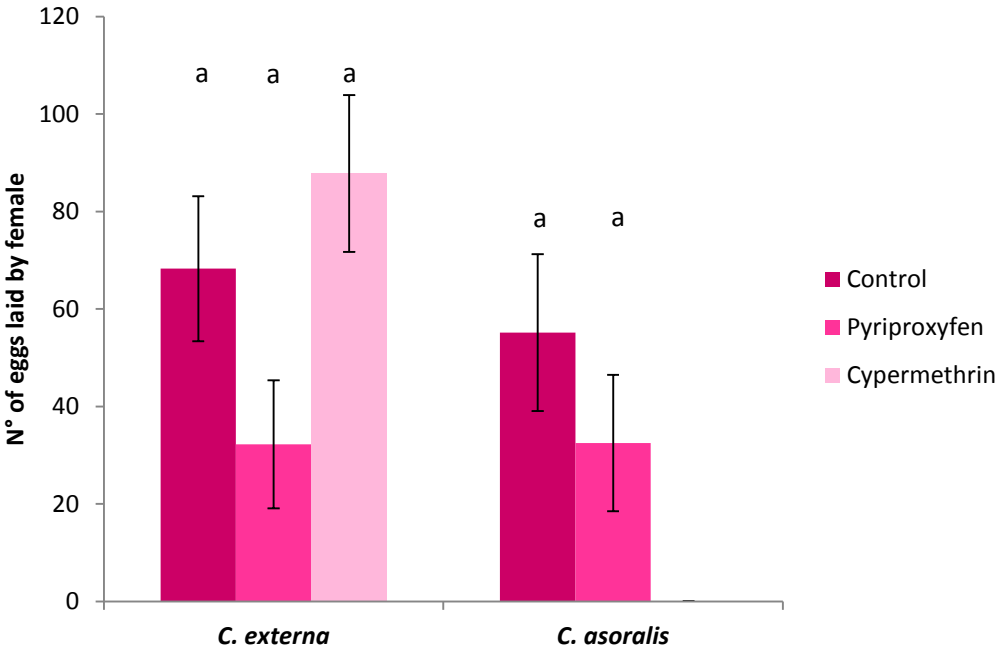

Fig. VI.6. Fecundity, measured as number of eggs laid by female. Data are mean ± SE. Different letters denote significant differences between treatments within each species ($P < 0.05$)

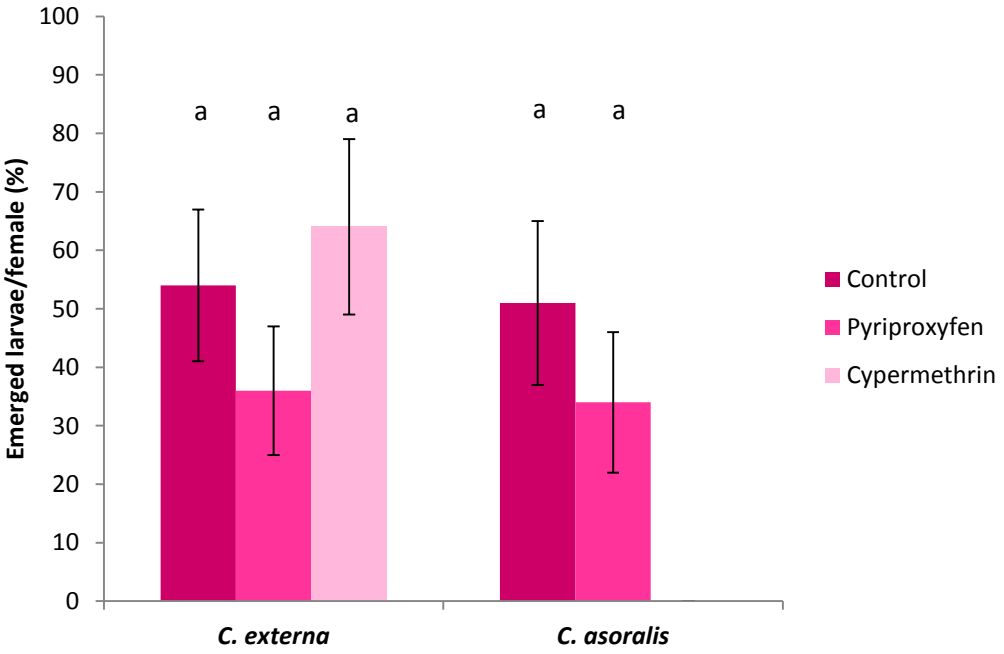

Fig. VI.7. Fertility as the percentage of number of emerged larvae. Data are *mean* ± SE. Different letters denote significant differences between treatments within each species ($P < 0.05$)

VI.3.2. Toxicity on treated pupal stage

VI.3.2.1. Mortality

No mortality was registered during the pupal stage of *C. externa*. On the contrary, low mortality was registered in *C. asoralis* individuals treated with cypermethrin (about 4% of pupae that could not reach the adult stage) (Cypermethrin mortality: *H = 1.9; df = 1; P = 0.31.* Data not shown).

VI.3.2.2. Developmental time from pupa to adult

Acetamiprid treatment significantly shortened the intermolt period in *C. asoralis* with respect to control. Same trend was observed for *C. externa* (pupa–adult period of *C. asoralis*: *H = 22.43; df = 3; P < 0.001*. Pupa–adult period of *C. externa*: *H = 63.96; df = 3; P < 0.001*) (Fig. VI.8).

The control treatment in *C. asoralis* had a longer pupa-adult period than *C. externa*. Acetamiprid also extended the period, however there were no differences between the species with pyriproxyfen or with cypermethrin (Pupa–adult period of control: *F = 9.31; df = 1,59; P = 0.003*. Pupa–adult period with pyriproxyfen: *H = 0.25; df = 1; P = 0.61*. Pupa–adult period with cypermethrin: *H = 1.63; df = 1; P = 0.2*. Pupa–adult period with acetamiprid: *H = 25.02; df = 1; P < 0.001*).

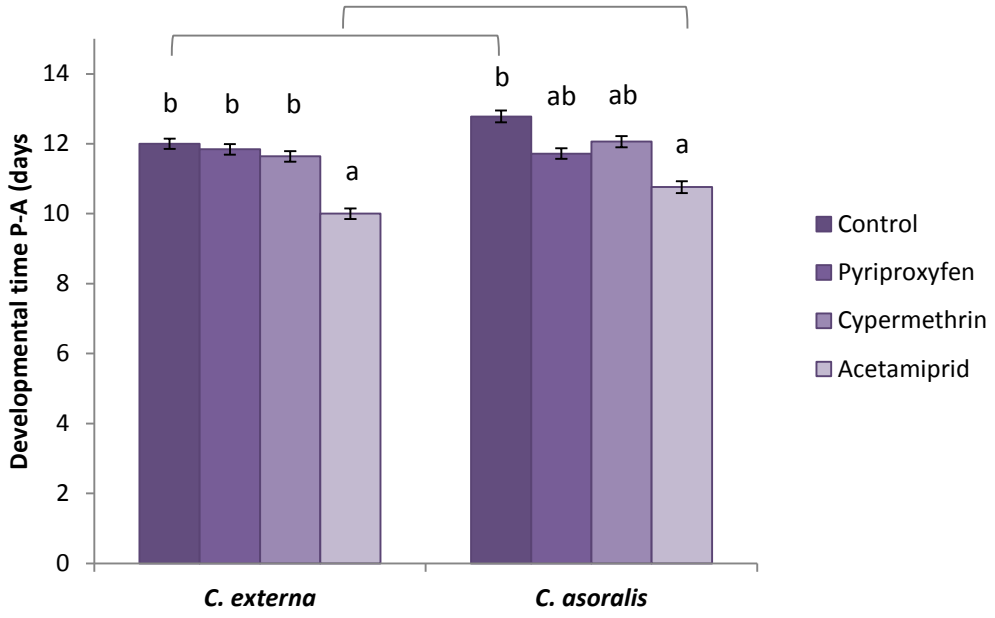

Fig. VI.8. Developmental time from pupa to adult (in days). Data are mean ± SE. Different letters denote significant differences between treatments within each species. Brackets mark the treatment that showed significant differences between species (*P < 0.05*)

VI.3.2.3. Preoviposition period

Even though adults were obtained after acetamiprid treated pupae, no female was able to oviposit after being paired with males. In all treatments, *C. asoralis* had a longer pre-oviposition period with respect to *C. externa* (Pre-oviposition period of control: *F = 26.18; df = 1,12; P = 0.0003*. Pre-oviposition period with pyriproxyfen: *F = 8.23; df = 1,8; P = 0.021*. Pre-oviposition period with cypermethrin: *F = 9.99; df = 1,10; P = 0.01*). Within each species, none of the treatments affected the mentioned period (Pre-oviposition period of *C. asoralis*: *F = 0.65; df = 2,14; P = 0.53*. Pre-oviposition period of *C. externa*: *F = 0.34; df = 2,14; P = 0.71*) (Fig. VI.9).

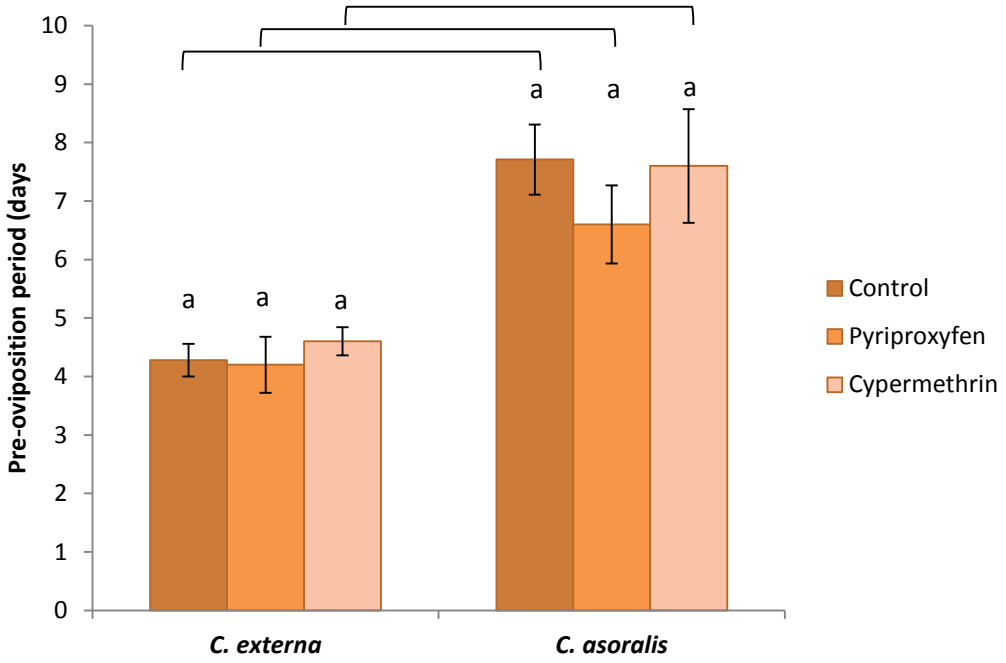

Fig. VI.9. Pre-oviposition period, time from the adult emergence until the first day of oviposition (in days). Data are mean ± SE. Different letters denote significant differences between treatments within each species. Brackets mark the treatment that showed significant differences between species (*P < 0.05*)

VI.3.2.4. Fecundity and fertility

Regarding the number of eggs laid, there were no significant differences between the different treatments within each species. Fertility, on the other hand, was lower in *C. asoralis* than in *C. externa* controls (comparison between species. Fecundity of control: *F = 1.85; df = 1,12; P = 0.19*. Fecundity of pyriproxyfen: *F = 0.6; df = 1,8; P = 0.46*. Fecundity of Cypermethrin: *F = 2.57; df = 1,10; P = 0.14*. Fertility of Control: *H = 5.63; df = 1; P = 0.017*. Fertility of Pyriproxyfen: *H = 3.23; df = 1; P = 0.07*. Fertility of Cypermethrin: *F = 0.63; df = 1,10; P = 0.44*).

The different treatments did not affect the fecundity or fertility in any species (*C. asoralis* fecundity: *F = 0.23; df = 2,14; P = 0.8*. *C. externa* fecundity: *F = 0.02; df =*

2,14; P = 0.98. *C. asoralis* fertility: *F = 0.19; df = 2,14; P = 0.82*. *C. externa* fertility: *F = 0.68; df = 2,14; P = 0.52*) (Fig. VI.10 and VI.11).

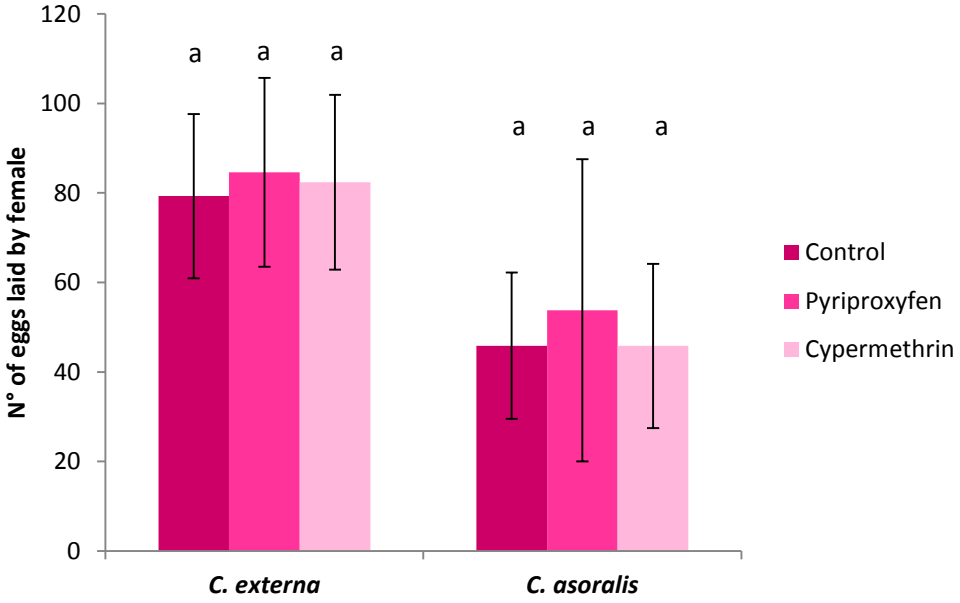

Fig. VI.10. Fecundity, as the number of eggs laid by female. Data are mean ± SE. Different letters denote significant differences between treatments within each species (*P < 0.05*)

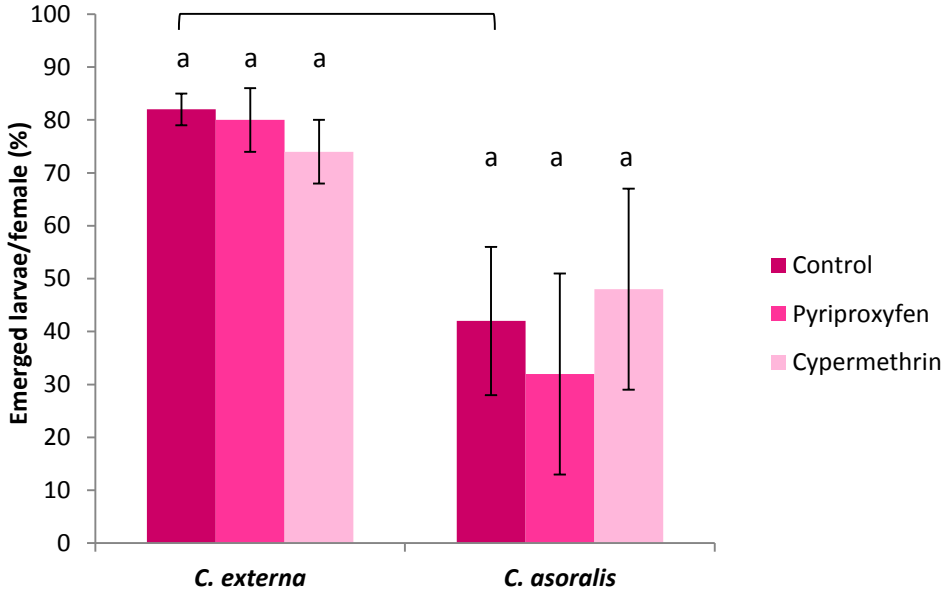

Fig. VI.11. Fertility, as the percentage of number of emerged larvae. Data are mean ± SE. Different letters denote significant differences between treatments within each species. Bracket marks the treatment that showed significant differences between species (*P < 0.05*)

VI.3.3. Susceptibility comparison between the treated stages

Susceptibility of larval stages and pupal stages was compared by analyzing the mortality values obtained from each topically treated stage was analyzed: mortality of topically treated larvae and mortality of topically treated pupae.

In both species, approximately 25% of individuals were affected by acetamiprid while the same insecticide did not kill any of the treated pupae. Also pyriproxyfen did not affect pupae and larvae did not have a considerable mortality in either species. Cypermethrin treatment in larvae of *C. asoralis* caused up to 85% of mortality; on the contrary *C. externa* larvae were not affected. Pupae of both species did not show significant mortalities (pyriproxyfen mortality in *C. asoralis*: $H = 2.5; df = 1; P = 0.11$ and *C. externa*: $H = 2.5; df = 1; P = 0.11$. Acetamiprid mortality in *C. asoralis*: $H = 4.5; df = 1; P = 0.033$ and *C. externa*: $H = 4.5; df = 1; P = 0.033$. Cypermethrin mortality in *C. asoralis*: $F = 143.33; df = 1,4; P < 0.001$ and *C. externa*: $H = 2.5; df = 1; P = 0.11$) (Fig. VI.12 and VI.13).

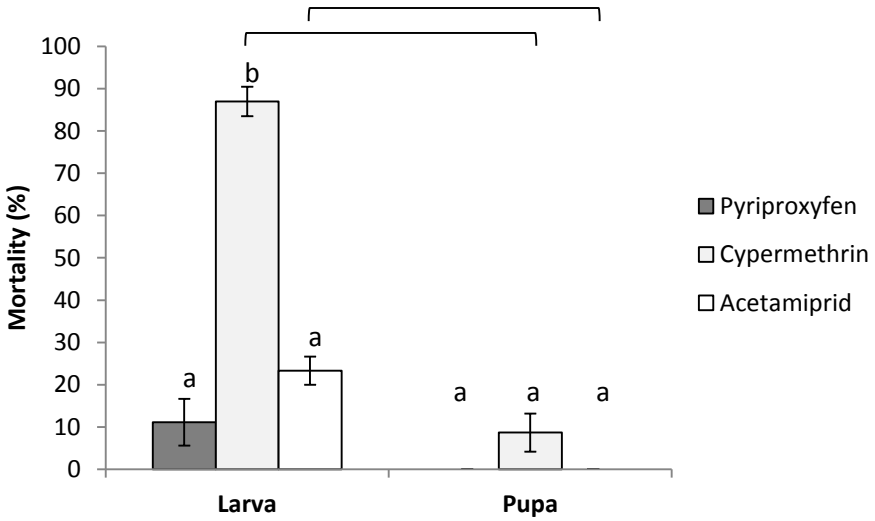

Fig. VI.11. Mortality percentage of different treated stages of *Chrysoperla asoralis.* Data are mean ± SE. Different letters denote significant differences between treatments within each species. Brackets mark the treatment that showed significant differences between species ($P < 0.05$)

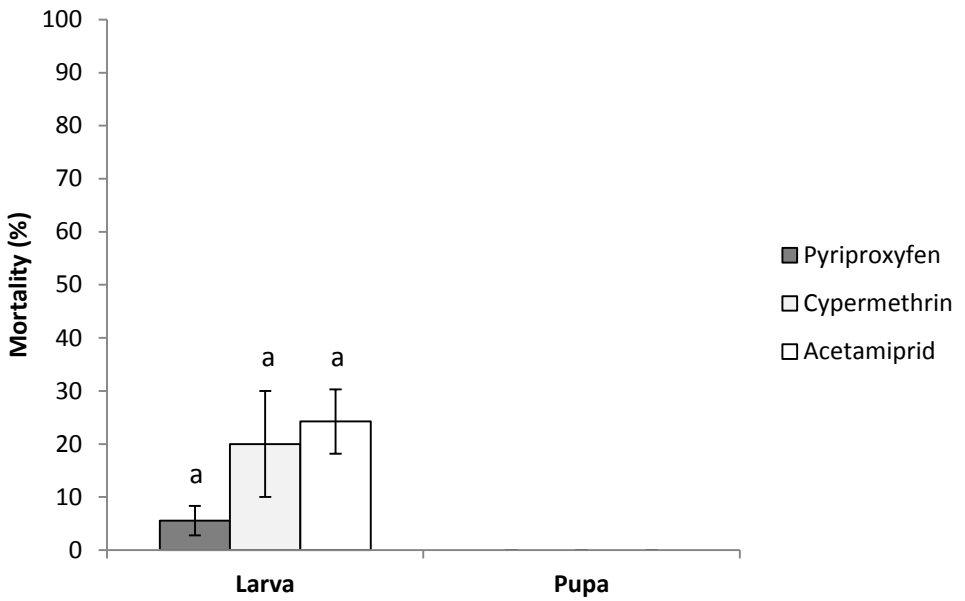

Fig. VI.12. Mortality percentage of different treated stages of *Chrysoperla externa.* Data are mean ± SE. Same letters denote no significant differences between treatments within each species *(P < 0.05*)

VI.4. Discussion

The present study revealed the higher susceptibility to insecticides in *C. asoralis* compared with *C. externa*. *C. asoralis* larvae that were able to reach the pupal stage, and that were treated with acetamiprid showed high mortality at the last stage. On the contrary, *C. externa* pupae had a very low mortality with acetamiprid but a 50% with cypermethrin. Pyriproxyfen was not toxic in larvae or pupae. Vivek *et al*. (2012), demonstrated a high toxicity to acetamiprid in *C. carnea* larvae, reaching up to 80% of mortality after 72 h of feeding treated prey. In the same study, they found that other biorational insecticide, the neonicotinoid thiamethoxam, had a similar effect. Shinde *et al.* (2009) observed *C. carnea* mortality from 53% to 80%, at 24 h and 72 h post-treatment, respectively, when the species were treated by residual exposure to acetamiprid. Likewise, field trials with acetamiprid on the population abundance of

Bemisia tabaci (Hemiptera: Aleyrodidae) predators, like *C. carnea* among others, demonstrated that the insecticide is highly toxic to the chrysopid with a decrease of 36% of the population (Naranjo & Akey 2005).

Regarding the high toxicity of cypermethrin registered in *C. asoralis* but not in *C. externa*, these results support the hypothesis that the second species presents a natural tolerance to cypermethrin, previously observed in this Thesis work (Chapter IV). On the other hand, there are studies which showed that toxicity of pyrethroids can be high on lacewings (Amarasekare & Shearer 2013; Rugno 2013) ; thus it becomes apparent that there exists the different susceptibility to cypermethrin between species and the active ingredient. The results obtained from larvae treated with pyriproxyfen, agree with those of *Ceraeochrysa cubana* Hagen (Neuroptera: Chrysopidae) where no lethal or sub-lethal effects (Rugno 2013) were recorded.

Results obtained in relation on the effect on developmental time from larva to pupa by chemical compounds, both species had an extension of the period with acetamiprid treatments. This could indicate a negative effect on the larval growth, showing an increased developmental time. Fogel *et al.* (2013) found that the developmental time of *E. connexa* from egg to adult was longer when eggs were treated with acetamiprid. Adult showed different preoviposition period between species in the control treatment: approximately 5 days in *C. externa* and 8 days in *C. asoralis*. Reproductive parameters did not differ between the two species. All treatments on larvae did not affect fecundity or fertility. In treatments on pupae it could be observed that this stage was much more tolerant to cypermethrin and acetamiprid than the larval stage, even though those insecticides were the most toxic to larvae. This may be due to the protection afforded by the silken cocoon build by the

third instar larvae, preventing the insecticide penetration (de Fátima *et al.* 2013; Rugno 2015).

The period to reach the adult stage has been shortened with acetamiprid in both species, and in *C. asoralis* in particular was affected pyriproxyfen and cypermethrin. These results could be related to a defence response of exposed organisms to insecticides in which the insect undergoes physiological mechanisms that accelerate the ecdysis. The ecdysial fluid accumulates between the old and new cuticle during the molting process. This fluid secretion does not only regulate the ecdysis but also acts in the protection of the body (Zhang *et al*. 2014).

Regarding the reproductive parameters, acetamiprid presented negative results, since females were infertile. These results are consistent with those obtained by Fogel *et al.* (2013) on *E. connexa,* where the reproductive capacity of adults from acetamiprid treated larvae, was reduced.

A longer preoviposition period was observed in controls of *C. asoralis* compared with *C. externa*, with 8 and 5 days, respectively. The fecundity and fertility were also unaffected and *C. asoralis* presented lower fertility than *C. externa* in the controls. The differences between species within the controls are expected to happen, being characteristics specific to the species. In this sense, *C. asoralis* has an intermolt time from larva to pupa and from pupa to adult, greater than *C. externa*. The pre-oviposition period is also greater and the number of neonates is lower than *C. externa*, but more studies are needed to elucidate differences between trials on larvae and pupae.

Another important factor to take into account in toxicity analyses of an organism -- either a pest or a natural enemy-- is the differential tolerance to

insecticides by the exposed developmental stage. For lacewings, as it was already discussed, pupal stages are the most resistant (Rugno 2015) and that could be corroborated in the present study.

CHAPTER VII

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Biological control is achieved through the use of organisms for maintaining a population of pests below the threshold level of economical damage. This approach is based on the already existing relationship—*e. g.*, predator-prey, host-parasitoid, host-entomopathogen— between different species within an agroecosystem (Eilenberg *et al*. 2001). Among the different types of biological control, the classical and neoclassical approach is based on the introduction of natural enemies of the pest species from outside the affected area to exert a control over exotic or native pests. Regulatory programs with these types of control have been widely used with success (Eilenberg *et al*. 2001; Van Driesche *et al*. 2007). In the more than 100 years of biological control, hundreds of species of exotic natural enemies have been imported, mass-reared and released (Greathead 1995; van Lenteren 2000, van Lenteren *et al.* 2003; Wratten & Gurr 2000). But after the haphazard introductions of not-native species causing dramatic outbreaks –most of all after Howarth (1983) stated this in his publication– restrictions on movements of biological material around the world were imposed. Testing of indigenous non-target species has rarely been applied as part of pre-release evaluation programmes for arthropod natural enemies (van Lenteren & Woets 1988; Waage 1997; Barrett *et al.* 2000). Today, and within the framework of Integrated Pest Management (IPM), the use of natural enemies against pests is part of this strategy; but the conservation or enhancement of endemic organisms is the way that IPM proposes. For this reason, as well as the regulation in chemical control, there is a need of regulation in biological control agents, *i.e.* in van Lenteren *et al*. (2003), a general framework for regulation of import and release of natural enemies was presented:

1. Characterization and identification of biological control agents: classical methods or molecular techniques, voucher specimens to be deposited, DNA fingerprinting in case of taxonomic problems;

2. Health risks: easier to determine for arthropod natural enemies than for chemical agents;

3. Environmental risks: identification of potential hazards posed to the environment based on collation of information, and data from experiments and observations, and a summary of the risks and benefits of the release of the exotic natural enemy in comparison with alternative control methods;

4. Efficacy: efficacy of biological control agents can be highly variable, particularly if no proper mass-rearing (van Lenteren & Tommasini 2003) and quality control methods (van Lenteren *et al*. 2003) are applied.

Conservation biological control was first developed after the damage done by chemicals to the beneficial organisms present in the area. Knowledge about the biology, ecology and behavior of both pests and natural enemies is critical, not only for the conservation of the latter but also for their enhancement. However, when pesticide applications are necessary, chemicals can be chosen to preserve natural enemies. But studies about the lethal and sublethal effects of insecticides on beneficials are necessary. Thankfully, not every natural enemy is killed by insecticides; moreover some species are tolerant or can develop resistance. One example is the Palearctic neuropteran *Chrysoperla carnea* which has developed resistance to pyrethroid insecticides that is associated with a high activity of detoxifying enzymes (Ishaaya & Casida 1981; Bashir & Crowder 1983; Pree *et al*. 1989; Pathan *et al.* 2008; Pathan *et al*. 2010; Sayyed *et al*. 2010). In Argentina, *C. externa* is present and it has

proven to be, at least, tolerant to broad-spectrum pesticides, because of its presence in fields of La Plata region with high rates of pesticide applications.

The first study of our work was to determine the susceptibility of lacewings of *C. externa* to pyrethroid insecticides, neonicotinoids, IGR and bioinsecticides, which are commonly used in the area. They were evaluated in the third larval and pupal stages. Specimens were collected in field crops and colonies were reared under controlled conditions. Laboratory and field colonies were compared. The susceptibility of both field populations of *C. externa* was the same as the laboratory colony, which was used as a reference. The toxicity of the pyrethroid cypermethrin was very low in both field populations and in the laboratory colony of *C. externa*. Topical application of 37.5 mg a.i./L (1.5 times the MFRC) produced a 60% of mortality in third instar larvae, while 100% of treated pupae survived. Cypermethrin did not affect the fecundity and fertility compared with controls. The toxicity of the neonicotinoid acetamiprid was higher than cypermethrin in third-instar larvae. The mortality was higher as the insecticide dose increased. The same tendency was observed for pupae from the surviving larvae, and very few individuals reached the adult stage. The bioinsecticide azadirachtin did not produce mortality in the third-instar larvae at any concentration; but 100% of mortality was registered for the pupal stage.

As a second approach and in accordance with the first results explained above, simultaneous application of cypermethrin with the enzyme synergists piperonyl butoxide (PBO) and S,S,S-tributyl phosphorotrithioate (DEF) increased the toxicity of the pyrethroid. These results suggest that the P450 mono-oxygenase enzymes and esterases could be involved in the biotransformation of cypermethrin. The enzymatic detoxification activity in the laboratory-reared individuals was the same in the field-

collected ones, which suggests that the detoxification process of the species remains even when they are not exposed to insecticides during several years.

Finally in the third study, the presence of *C. asoralis* and *C. argentina* was reported for the first time in the Horticultural Belt of La Plata, and in association to sweet pepper crops *Capsicum annuum*. This is of a great importance as until now only *C. externa* was identified in the area. *C. asoralis* was the species that could be reared in laboratory for experimental purposes. Third-instar larvae of *C. asoralis* were more susceptible to cypermethrin and acetamiprid compared to *C. externa* larvae. The IGR pyriproxyfen was not toxic to either of the two species. On the other hand, treatment of these insecticides on pupae demonstrated that this stage is more tolerant to topical applications compared to larval instars. *C. externa* exhibits a natural tolerance to cypermethrin, probably due to a higher P450 mono-oxygenase and esterase enzyme detoxification activity. This tolerance, as a characteristic of the species, enables that even laboratory individuals without exposure to insecticides could survive to field applications of pyrethroids.

As future perspectives, the attribution of a natural tolerance in *C. externa* to pyrethroid insecticides is of utmost importance in mass-rearing programs of this predator. As a consequence, these useful predators would then be able to survive the applications and/or insecticide residuals in the field, during field releases for biological control, even if they were not exposed during their rearing. For species with an important role as biological control agents, but with no tolerance to pesticides, genetic engineering of resistant strains could be a promising and ambitious topic. It should be of great importance to apply molecular characterization to natural enemies in order to make predictions about adaptive differences between species or populations of the

same species (Fisher *et al*. 1999). Molecular biology could provide us with information about host range, climatic tolerances, insecticide resistance, and thus could increase the success of the selection of a potentially suitable natural enemy. This is very important in the classical approach of biological control, because of the differences that geographic races or biotypes of the natural enemy could have and could be critical for the establishment in the new environment. But, in the framework of this dissertation, where a tolerant natural enemy was identified, and the former is endemic from the area, we believe that the biological control approach by conservation could be improved. For instance, molecular characterization of *C. externa*, which proved to be tolerant to some insecticides, could be useful for the development of genetically engineered *C. asoralis* in order to keep them in the agroecosystem. Although IPM demands the use of chemicals as a last strategic option, it is a real-world concern that several pests are not controlled by any other type than chemical control. In accordance with this, a natural enemy that could survive this real environmental scenario should be a promising agent and a new challenge for the future of biological control.

It should also be remarked that negative effects of the release of exotic natural enemies, bringing the biodiversity of the area at risk, have been reported (Roversi *et al.* 2014). The effects could be the direct attack on non-target organisms, indirect effects on non-targets, dispersal of the agent to other areas, or change of the relationship between a control agent and a native species (Simberloff 2012). For this reason, and with the knowledge of the existence of native biocontrol agents, their conservation should be a major concern.

It is also demanding to generate more knowledge of the arthropod fauna that is present in the area of La Plata through multidisciplinary studies, and in turn this should

increase the range of alternatives of control. Besides, a transference program should be encouraged, so the growers from the area can apply IPM strategies in the field.

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SUMMARY

Integrated pest management (IPM) is a decision-making system for selecting suitable strategies for the control of phytophagous pests. This management is based on the analysis of cost-benefit, taking into account the interest and impact on farmers, society and the environment. Since the beginning of its definition, IPM has come a long way adding the concept of “plant protection methods” instead of “pest control techniques” and the notion of “ecological justification” instead of economic justification. Among the different strategies implemented by IPM, some of them are: cultural control (mechanical tillage methods, soil management, crop rotation, etc.), genetically modified cropping systems (use of pest-resistant crops and plant breeding), ethological control (autocidal control, sex pheromones, repellents, etc.), chemical control and biological control. Chemical control has been promoted worldwide during decades until negative impacts of this strategy came to light, and within the framework of IPM this type of control is used only when other strategies do not yield satisfactory results and with selective pesticides. Biological control is accomplished through the use of organisms to maintain the population of phytophagous pests below the Economic Injury Level. The control is based on the relationships between different organisms in agroecosystems (predator-prey, host-parasitoid, and host-entomopathogen). Pest control in Argentina is mainly based on the use of broad-spectrum pesticides, especially organophosphates, carbamates and pyrethroids. Extensive use and often disregard and/or irresponsible use of these pesticides has led to the emergence of resistance in many pest populations. As a result, control fails and this lead to bad practices, such as increasing the dose or frequency of pesticide applications. Pesticide resistance by pests has been extensively studied because of its importance in economical terms, since it reduces the effectiveness of control of a chemical

treatment. Other consequences of these activities are the negative effects on populations of natural enemies. But it is also known that natural enemies can develop resistance, which would mean an opportunity to integrate the biological and chemical control in agro-ecosystems where pests have to be controlled with this type of insecticides.

The Horticultural Belt of La Plata (*i.e.*, Cinturón Hortícola Platense [CHP]) is a very important productive region which has been developed in an economical, technological and commercial way, in a constant manner since its origin around the capital of Buenos Aires Province. In the last 24 years, this development adds a qualitative distinction to the quantitative one, with a high quality product and an extension in the supply period. On the other hand, the use of greenhouse production starts in the '80s with an important increase in the '90s, boosting the value of this area not only in a regional level (with a 90% of greenhouses in the province), but also in a national level (50% of greenhouses in the country).

The genus *Chrysoperla* Steinmann (Neuroptera: Chrysopidae) is the most abundant within the family, and it is used as a biological control agent in IPM programs. *Chrysoperla carnea* Stephens, widely distributed in the Holarctic region, is the most commercialized species as a biological control agent. In Argentina, there are four species of *Chrysoperla*: *C. externa* Hagen, *C. asoralis* Banks, *C. argentina* González Olazo & Reguilón, and *C. defreitasi* Brooks. In this work, *C. externa* was evaluated as it is present in the studying area. It presents a high adaptability to different environmental conditions, high reproductive potential, efficient search capability, and it is associated with various agricultural crops. The larvae of this species are effective natural enemies of aphids, whiteflies, thrips, mites and moths. These features make *C.*

externa a good candidate for biological control and in recent years, it has promoted the mass rearing and subsequent release in the field of this species, in several South American countries. Previous studies have shown tolerance to insecticides in this species so it could be combined in programs in which both chemical and biological control are required.

C. externa proved to be a promising natural enemy that can be used in IPM programs. The species *C. externa* presents natural tolerance to the insecticide cypermethrin, probably because of a high detoxifying action by P450 enzymes and esterases. This self-tolerance of the species is present in laboratory strains without previous exposure to insecticides.

However, acetamiprid which was considered a biorational insecticide, proved to be toxic to *C. externa.* Azadirachtin did not cause mortality to treated larvae but the 100% of pupae did not reach the adult stage. The insect growth regulator (IGR) pyriproxyfen on the contrary was harmless to the species.

As another goal in this work, the distribution of the species *C. argentina* and *C. asoralis* in the country, has been expanded, citing them in the CHP, and in association with sweet pepper crops, for the first time. For that reason, *C. asoralis* –the most abundant of the two new species– was evaluated as well. Bioassays with insecticides on the species *C. asoralis* compared with *C. externa*, showed a greater susceptibility to insecticides in the first one, and regarding cypermethrin the mortality reached a 100%. These results reinforce the hypothesis of a natural tolerance to pyrethroids in *C. externa*, exclusively of that species.

This feature is of great importance in programs of *C. externa* mass rearing, since at the time of release in the field, individuals would be able to survive applications and/or residues of pesticides, without having been exposed during their rearing.

SAMENVATTING

Het doel van geïntegreerde gewasbescherming (IPM) is de selectie van geschikte strategieën voor de bestrijding van fytofage insectenplagen. Deze selectie is gebaseerd op een analyse van de kosten en de voordelen, waarbij de belangen voor en de impact op de landbouwers, de samenleving en het milieu worden meegenomen. Sinds het ontstaan heeft IPM ervoor gezorgd dat er een focus kwam op de bescherming van de plant of het gewas, in plaats van het doden van insecten, en dit op een ecologisch verantwoorde wijze in plaats van op een louter economische. Tot de verschillende mogelijke IPM strategieën behoren onder andere cultuurtechnische methoden (bv. mechanische grondbewerking, bodembeheer, gewasrotatie), genetisch gemodificeerde gewassystemen (bv. het gebruik van plaagresistente gewassen en veredeling), ethologische controle (bv. autocidale beheersing, sexferomonen, insectenwerende middelen), chemische bestrijding en biologische bestrijding. De chemische bestrijding is wereldwijd decennialang gepromoot tot de negatieve gevolgen duidelijk werden. Binnen het kader van IPM wordt dit type van bestrijding, gebruik makende van selectieve pesticiden, enkel nog aangewend wanneer andere strategieën geen voldoende resultaten leveren. Biologische bestrijding houdt in dat andere organismen worden gebruikt om de populaties van fytofage insectenplagen onder de economische schadedrempel te houden. Deze bestrijding is gebaseerd op de relatie tussen verschillende organismen in een agro-ecosysteem (predator-prooi, gastheer-parasitoïde en gastheer-entomopathogeen).

De bestrijding van plaaginsecten in Argentinië is hoofdzakelijk gebaseerd op het gebruik van breedspectrum pesticiden, met name organofosfaten, carbamaten en pyrethroïden. Het intensieve en vaak ook onverantwoorde gebruik van deze pesticiden heeft geleid tot het opkomen van resistentie in veel plaagpopulaties. Dit heeft tot

gevolg dat de bestrijding mislukt en het leidt vaak tot te vermijden praktijken, zoals het verhogen van dosissen en de frequentie van pesticidengebruik. Pesticidenresistentie bij insectenplagen is uitgebreid onderzocht vanwege het economische belang, aangezien het de effectiviteit van de chemische bestrijding vermindert. Andere gevolgen van het frequent en intensief gebruik van deze pesticiden zijn de negatieve gevolgen voor populaties van natuurlijke vijanden. Het is echter geweten dat ook deze natuurlijke vijanden resistentie kunnen ontwikkelen tegen deze producten, waardoor er toch een mogelijkheid ontstaat om de biologische en chemische bestrijding te integreren in agro-ecosystemen en deze plagen te bestrijden met deze insecticiden.

De “Horticultural Belt” van La Plata (Cinturón Hortícola Platense [CHP]) is een erg belangrijke en productieve regio rond de hoofdstad Buenos Aires die op economisch, technologisch en commercieel vlak constant verder ontwikkeld is sinds het ontstaan. Gedurende de laatste 24 jaar is er bij deze ontwikkeling ook een focus gekomen op het kwalitatieve, naast het kwantitatieve, aspect met een kwalitatief eindproduct en een beschikbaarheid over een langere periode. De productie in serres vatte aan in de jaren ’80, met een sterke stijging in de jaren ’90, waardoor de waarde van deze regio nog vergrootte, niet enkel op een regionaal niveau (90% van alle serreteelt in de provincie), maar ook op een nationaal niveau (50% van alle serreteelt).

Het geslacht *Chrysoperla* Steinmann (Neuroptera: Chrysopidae) is het vaakst voorkomend binnen de familie, en het wordt gebruikt voor biologische bestrijding in IPM programma’s. *Chrysoperla carnea* Stephens, wijd verspreid in de Holarctische regio, is de meest gecommercialiseerde soort in deze context. In Argentinië worden vier *Chrysoperla* species aangetroffen: *C. externa* Hagen, *C. asoralis* Banks, *C. argentina* González Olazo & Reguilón, en *C. defreitasi* Brooks. In dit onderzoek werd *C. externa*

gebruikt aangezien deze aanwezig is in de onderzoeksregio. Het bezit de capaciteit om zich aan te passen aan verschillende milieuomstandigheden, het heeft een hoge reproductiecapaciteit, het is een efficiënte zoeker en is geassocieerd met verschillende landbouwgewassen. De larven van deze soort zijn effectieve natuurlijke vijanden van bladluizen, wittevliegen, thrips, mijten en nachtvlinders. Deze eigenschappen maken van *C. externa* een goeie kandidaat voor biologische controle. Het wordt op dit moment dan ook in heel wat Zuid-Amerikaanse landen op grote schaal gekweekt en losgelaten in de gewassen. Eerdere studies hebben tolerantie voor insecticiden aangetoond in dit species, dus is het ook geschikt voor programma's waarin zowel chemische en biologische bestrijding vereist is. *C. externa* bleek een veelbelovende natuurlijke vijand te zijn die gebruikt kan worden in IPM systemen. De soort vertoont, waarschijnlijk dankzij detoxificatie door P450 mono-oxygenase enzymen en esterasen, een natuurlijke tolerantie voor het insecticide cypermethrin. Deze soort-eigen tolerantie is aanwezig in laboratoriumstammen, ook zonder eerdere blootstelling aan insecticiden. Niettemin bleek acetamiprid, dat gezien wordt als een 'biorationeel insecticide', toxisch voor *C. externa*. Azadirachtin veroorzaakte geen directe mortaliteit bij larven, maar 100% van de poppen bereikten het volwassen stadium niet. De insectengroeiregulator pyriproxyfen bleek dan weer onschadelijk voor deze soort.

Een ander doel van dit onderzoek was het evalueren van de distributie van *C. argentina* en *C. asoralis*, die voor het eerst werden aangetroffen in de CHP regio, in paprikagewassen. Hierom werd *C. asoralis*, het vaakst voorkomende van beide species, ook geëvalueerd in dit onderzoek. In vergelijking met *C. externa* bleek *C. asoralis*, op basis van biotoetsen met de insecticiden, een grotere gevoeligheid te vertonen tegen deze insecticiden. Voor cypermethrin werd een mortaliteit van 100% vastgesteld. Deze

resultaten versterken de hypothese dat de natuurlijke tolerantie voor pyrethroïde insecticiden bij *C. externa* exclusief is voor deze soort.

Deze eigenschap is van groot belang voor het gebruik van *C. externa* in IPM programma's en de massakweek, aangezien de insecten op het moment van loslaten in het veld, zonder eerdere blootstelling tijdens het opkweken, geen last zullen hebben van toepassing van deze pesticiden of overgebleven residu's.

RESUMEN

El Manejo Integrado de Plagas (MIP) es un sistema de toma de decisiones para la selección de estrategias de manera armoniosa para el control de artrópodos fitófagos. Esta estrategia de manejo se basa en el análisis del costo/beneficio, teniendo en cuenta los intereses y el impacto en los productores, la sociedad y el medio ambiente. Desde los comienzos de su implementación, el MIP ha recorrido un largo camino y el concepto de "técnicas de control de plagas" fue reemplazado por "métodos de protección vegetal", así como también se introdujo la noción de "justificación ecológica" en vez de justificación económica. Entre las diferentes estrategias implementadas por el MIP, se encuentran: el control cultural (métodos mecánicos de labranza, manejo de suelos, rotación de cultivos, etc.), el control fitogenético (uso de cultivos resistentes a plagas y mejoramiento genético), el control etológico (control autocida, uso de feromonas sexuales, repelentes, etc.), el control químico y el control biológico. El control químico ha sido promovido durante décadas hasta que sus efectos negativos salieron a la luz, y dentro del marco del MIP este tipo de control debería ser aplicado solo luego de que otras estrategias no lleguen a resultados satisfactorios, y con la utilización de insecticidas selectivos. El control biológico se lleva a cabo a través de la utilización de organismos para mantener la población de un artrópodo fitófago por debajo del nivel de daño económico. Esta medida de control se basa en las relaciones existentes entre diferentes especies en los agroecosistemas (relación depredador-presa y huésped-parasitoide, huésped-entomopatógeno). El control de plagas en Argentina se basa principalmente en el uso de plaguicidas de amplio espectro, principalmente organofosforados, carbamatos y piretroides. El uso extensivo y muchas veces el desconocimiento y/o el uso irresponsable de estos plaguicidas ha llevado al surgimiento de resistencia en muchas poblaciones de plagas. Como

consecuencia, ocurren fallas de control que a su vez conducen a malas prácticas, como el aumento de las dosis o de la frecuencia de las aplicaciones. La resistencia a plaguicidas por parte de las plagas ha sido estudiada extensamente por su importancia en términos económicos, ya que reduce la efectividad de control de un tratamiento químico. Otra consecuencia de estas actividades son los efectos negativos sobre las poblaciones de enemigos naturales. Pero también es sabido que los enemigos naturales pueden desarrollar resistencia, lo cual significaría una oportunidad para integrar el control biológico y químico en agroecosistemas donde las plagas deben controlarse indefectiblemente con este tipo de insecticidas.

En Argentina, el Cinturón Hortícola Platense (CHP) es una región altamente productiva y que se ha desarrollado exitosamente a nivel económico, tecnológico y comercial, de una manera ininterrumpida desde sus orígenes alrededor de la Capital de la Provincia de Buenos Aires. En los últimos 24 años, a este desarrollo cuantitativo se le sumó el componente cualitativo, con una mayor calidad del producto y una extensión en el período de oferta. Por otro lado, la producción bajo invernáculo comenzó en la década de los ‘80s con un auge en los ‘90s, dándole importancia al área no sólo a nivel regional (con un 90% de la producción bajo invernáculo en la Zona Bonaerense) sino a nivel nacional (con un 50% de producción bajo invernáculo en Argentina).

El género *Chrysoperla* Steinmann (Neuróptera: Chrysopidae) es el más importante dentro de los neurópteros, debido a la utilización de estos especímenes como agentes de control biológico en programas de MIP. *Chrysoperla carnea* Stephens, ampliamente distribuida en la zona Holártica, es la especie más comercializada como agente de control biológico, de esta familia. En Argentina, se han registrado cuatro especies del género *Chrysoperla*: *C. externa* Hagen, *C. asoralis* Banks, *C. argentina* González Olazo &

Reguilón y *C. defreitasi* Brooks. En el presente trabajo, se evalúa a la especie *C. externa* por estar presente en el área de estudio del CHP. *C. externa* presenta una alta adaptabilidad a diferentes condiciones ambientales, alto potencial reproductivo, eficiente capacidad de búsqueda de la presa y está asociada a varios cultivos agrícolas. Las larvas de esta especie son enemigos naturales efectivos de áfidos, mosca blanca, trips, ácaros y lepidópteros. Estas características hacen de *C. externa* un buen candidato para control biológico. Durante los últimos años, ha sido promovida su cría masiva y consecuente liberación en campo, en varios países de Sudamérica. Estudios previos han demostrado la tolerancia a insecticidas en esta especie por lo que podría exitosa en programas en los que se requieran tanto control químico como biológico.

C. externa demostró ser un enemigo natural prometedor y que puede ser utilizado tanto en programas de MIP, como en agroecosistemas donde el uso de insecticidas neurotóxicos es inevitable. La especie *C. externa* presenta tolerancia natural al insecticida cipermetrina, probablemente por una mayor acción detoxificante por parte de enzimas P450 y esterasas. Esta tolerancia propia de la especie, hace que incluso individuos de laboratorio sin exposición a insecticidas, puedan sobrevivir a aplicaciones de piretroides. Sin embargo, acetamiprid que es considerado como un insecticida biorracional, fue altamente tóxico. Azadiractina no produjo mortalidad en las larvas tratadas pero en el estado pupal ningún individuo pudo alcanzar el estado adulto, por lo cual hubo un 100% de mortalidad. El regulador de crecimiento piriproxifén, por el contrario, resultó no tóxico para la especie.

Por otro lado, se amplió la distribución de las especies *C. argentina* y *C. asoralis* en el país, citándolas por primera vez en el CHP, provincia de Buenos Aires, y en asociación con el cultivo de pimiento. *C. asoralis* (la más abundante de las dos nuevas especies

citadas) se incorporó a las evaluaciones las cuales permitieron observar una mayor susceptibilidad a los insecticidas cipermetrina, piriproxifén y acetamiprid, en la primera. Estos resultados refuerzan la hipótesis de una tolerancia natural a piretroides en *C. externa*, exclusiva de la especie.

Dicha característica es de suma importancia en programas de cría masiva de este depredador, ya que al momento de la liberación en campo, los individuos serían capaces de sobrevivir aplicaciones y/o presencia de residuos de insecticidas, sin haber sido expuestas a los mismos durante su cría.

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EDUCATION

- High School degree with Orientation in Natural Sciences at Liceo Víctor Mercante of La Plata - Argentina
- **University degree**: Biology with Orientation in Zoology (5 years career). Faculty of Natural Sciences and Museum. University of La Plata (UNLP) – Argentina. Year 2011. Mean classification: **8.23/10**

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- 62nd International Symposium on Crop Protection. Ghent-Belgium, May 18th, 2010. “Compatibility of a *Melia azaderach* extract with *Eriopis connexa* (Coleoptera: Coccinellidae)”. **Haramboure M**, Mirande L, Smagghe G, Pineda S, Schneider M I. (Poster)

PRESENT POSSITION

- PhD student **(2011 – actual).**
- Thesis: "Evaluation of the tolerance and susceptibility to conventional and biorational pesticides in *Chrysoperla externa* (Neuroptera: Chrysopidae) and *C. asoralis* (Neuroptera: Chrysopidae)".

  JOINT PhD between Faculty of Natural Sciences and Museum. National University of La Plata (UNLP - Argentina) & Faculty of Bioscience Engineering. Ghent University (UGent - Belgium).

GRANTS

• Training Scholarship for university students awarded by the Scientific Research Commission (CIC). Topic: **"Techniques for mass rearing of *Chrysoperla externa*, with a view to marketing as an agent of Biological Pest Control".**

Supervisor: Dr. Marcela Inés Schneider.

Place: CEPAVE (UNLP/CONICET)

Period: 10/01/2010 to 03/31/2011.

• Postgraduate Scholarship from National Scientific and Technical Research Council (CONICET - Argentina) from April 1, 2011. Topic: **"Evaluation of the tolerance and susceptibility to conventional and biorational pesticides in *Chrysoperla externa* (Neuroptera: Chrysopidae) and *C. asoralis* (Neuroptera: Chrysopidae)".**

Supervisor: Dr. Marcela Inés Schneider (UNLP, La Plata, Buenos Aires. Argentina).

Co-Supervisor: Dr. Raúl Adolfo Alzogaray (UNSAM, San Martín, Buenos Aires. Argentina).

Place: CEPAVE (UNLP/CONICET)

Period: 01/04/2011 to 31/03/2016.

• EuroTANGO II Scholarship for a "Sandwich PhD" in Ghent University, Belgium. Coordinator Institution: Universitat Politécnica de Valencia in the framework of **Erasmus Mundus** Action 2-Strand 1 program.

Topic: "**Evaluation of resistant mechanisms to pesticides in the generalist predator Chrysoperla externa (Neuroptera: Chrysopidae)".**

Promotor UGhent: Prof. dr. ir. Guy Smagghe.

Promotor UNLP: Dra. Ing. Agr. Marcela Inés Schneider. Co-promotor: Dr. Raúl Adolfo Alzogaray.

Working period: 9/5/2013 to 31/10/2013.

Place: Faculty of Bioscience Engineering, Department of Crop Protection, Lab. of Agrozoology.

• Postdoctoral Scholarship from National Scientific and Technical Research Council (CONICET - Argentina) from April 1, 2016. Topic: **"Microsporidia and protists entomopathogens associated to the pollinators *Bombus sp.* and *Apis mellifera* (Hymenoptera: Apidae) from horticultural crops of northwestern Pampas Region".**

Supervisor: Dr. Carlos Lange (UNLP, La Plata, Buenos Aires. Argentina).

Co-Supervisor: Prof. Dr. ir. Guy Smagghe (UGent, Ghent. Belgium).

Place: CEPAVE (UNLP/CONICET)

Period: 01/04/2016 to 31/03/2018.

RESEARCH PROJECTS

Collaborator in the Project ANPCyT (FONCYT) Ministry of Sciences, Technology and Productive Innovation – Argentina. PICT 2011, N° 1752, Res. N°: 140-12. "Natural enemies and low environmental impact pesticides for pest control in horticultural crops. Implications in the Sustainable Agriculture." Director: Dra. Marcela Inés Schneider.

2013-2015. FP7-PEOPLE-2012-IRSES. Marie Curie Actions — International Research Staff Exchange Scheme (IRSES). ASCII— "Ameliorating the sustainable control of invasive insects." Director: Emilio Guerrieri (Consiglio Nazionale delle Ricerche Istituto per la Protezione delle Piante; Italy).

ACADEMIC SOCIETY

Member of the Argentinean Entomological Society (**SEA**). 2011-2015.

POSTGRADUATE COURSES

"**Agroecology**". Professor Ing. Agr. Santiago J. Sarandón. Faculty of Agrarian and Forestry Sciences (UNLP). Year 2011. Credit hours: 64 h. Approved: 9 (nine)

"**Management of research quality**". CONICET La Plata. Year 2011. Credit hours: 20 h. Approved: 10 (ten)

"**Vegetal protection and ecotoxicology**". Professor Dra. Alicia E. Ronco. Faculty of Agrarian and Forestry Sciences (UNLP). Year 2011. Credit hours: 45 h. Approved: 9 (nine)

"**Design of experiments**". Professor Ing. Agr. María Urrutia. Faculty of Agrarian and Forestry Sciences (UNLP). Year 2012. Credit hours: 60 h. Approved: 9 (nine)

"**Integrated pest management**". Professor Ing. Agr. Daniel Leiva INTA Pergamino and Ing. Agr. Susana B. Padín. FCAyF-UNLP. Faculty of Agrarian and Forestry Sciences (UNLP). Year 2012. Credit hours: 45 h. Approved: 7 (seven)

"**Pesticides: modes of action**". Professor Dr. Eduardo Puricelli. Faculty of Agrarian and Forestry Sciences (UNLP). Year 2012. Credit hours: 45 h. Approved: 8 (eight)

"**Applied Biostatistic with R program**". Professor Dr. Arnaldo Mangeaud. Faculty of Exact Physics and Natural Sciences (National University of Córdoba). Year 2014. Credit hours: 90 h. Approved: 10 (ten)

LANGUAGES

Spanish: mother tongue.

English: full professional proficiency (ILR)

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