Estudio de la bioecologia de la familia Chrysopidae (Insecta: Neuroptera) desde la perspectiva de su incremento y conservación en el olivar

Bioecological study of the Chrysopidae family (Insecta: Neuroptera) in olive orchards from a population growth and conservation perspective

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Editor: Editorial de la Universidad de Granada
Autor: Mario Porcel Vílchez
D.L.: GR 2334-2012
ISBN: 978-84-9028-162-8

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Esta Tesis Doctoral ha sido realizada en el Grupo de Protección Vegetal perteneciente al Departamento de Protección Ambiental de la Estación Experimental del Zaidín, gracias a una Beca Predoctoral de formación de doctores concedida por la Agencia Estatal Consejo Superior de Investigaciones (CSIC) del Ministerio de Educación y Ciencia.

Contents

Summary

Green lacewings (Neuroptera: Chrysopidae) are among the most common natural enemies of pest insects present in almost all the agricultural ecosystems worldwide. In olive orchards, chrysopids are known to consume several insects of economic importance, however, their main beneficial action has been associated to the prevention of the damage caused by the olive moth, Prays oleae (Bernard). Thus, the conservation and enhancement of this group of species in olive cropping may result in an increased natural control of pest populations and in a reduction of the dependence on other control methods such as chemical insecticides. In this thesis, several aspects of chrysopid conservation biological control in olive orchards have been addressed aiming at improving green lacewings presence and diversity.

Chapter 4 describes an experimental setup using the automated computerized video tracking EthoVision XT for mobility behavior experiments on *Chrysoperla carnea s.l.* This methodology was able to identify different behavioral patterns, corresponding to differences in searching and feeding behavior, based on several movement parameters accurately calculated by the EthoVision system. The information provided by this test indicates that this methodology may be applied in a variety of behavioral experiments. In Chapter 5, the compatibility with the chrysopid *C. carnea s.l.* of a novel pest control system used in olive orchards against the olive fruit fly, kaolin clay particle films, was investigated by assessing its effect on this predator at different levels. Kaolin did not produce acute mortality to immature stages of *C. carnea s.l.*. Nevertheless, mobility assessment experiment using the methodology described in Chapter 5 indicated that kaolin-covered larvae suffered mobility impediments, and that the movement ability on kaolin-covered surfaces was also reduced. In addition, larvae fell off easily from kaolin-treated olive leaves which led to hypothesize that the disruption of the movement capacity and dislodgement from the plant surface may be the principal negative effects of particle film on *C. carnea s.l.* larvae. Adult *C. carnea s.l.* preferred kaolin-

treated leaves for oviposition, however, an experiment carried out in field conditions revealed no preference towards treated trees.

Several field experiments were carried out in 10 different olive orchards in different years with the objective of establishing the effect of insecticide use and the presence of vegetation cover (VC) on chrysopid abundance and diversity as well as the overall effect of agricultural management type (Chapters 6 and 7). Thus, three orchards with different level of management intensification were sampled in 1999 and 2000 and 9 orchards (3 per type of management; conventional, integrated and organic) in the year 2003. The use of the pesticide dimethoate caused no effect on larval and adult stages of the most abundant species, *C. carnea s.l.* but influenced negatively *Dichochrysa* spp. causing a significant reduction in their populations. These findings suggest that species of the Chrysopidae family may differ significantly in their response to insecticides. It was also concluded that management intensification in olive orchards is related to a loss in chrysopids species biodiversity fostering *C. carnea s.l.* dominance. Due to the relationship detected between the lack of weed suppression and the increased presence of *C. carnea s.l.* adults, in the years 1999 and 2000, the effect of managed VC was investigated separately from other management factors during 2009 and 2010. The VC, present from early spring until the month in June, increased the abundance of the most important species in Andalusian olive orchards, *C. carnea s.l.* and *Dichochrysa prasina* (Burmeister), and also contributed to a slight increment in the diversity of chrysopid species. The increased *C. carnea s.l.* adults associated to vegetation cover produced higher number of *C. carnea s.l.* larvae on olive trees that coincided temporally with *P. oleae* oviposition onset. By contrary *Dichochrysa* spp. larvae did not increase in the presence of VC.

Finally, a marking technique, described in Chapter 8, was developed to be used in mark-release-recapture experiments in order to study chrysopid adult movements with the objective of improving the knowledge for conservation biological control in olive orchards. *C. carnea s.l.* adults were marked permanently producing a change in color identifiably mainly in the abdomen by feeding the larvae with an oil soluble dye, Sudan Red 7B,

incorporated to an artificial meridic diet. Optimal dye concentrations were established in order to guarantee both an adequate marking level and an acceptable larval survival rate with no substantial effects on larval development and diet consumption, and adult fecundity and flight performance.

Resumen

Los crisópidos (Neuroptera: Chrysopidae) se encuentran entre los enemigos naturales más habituales de insectos que provocan plagas y están presentes en casi la totalidad de los ecosistemas agrícolas a nivel mundial. En el olivar, los crisópidos desarrollan una actividad depredadora sobre varias plagas relevantes desde el punto de vista económico pero su principal acción beneficiosa radica en la prevención del daño causado por la polilla del olivo *Prays oleae* (Bernard). De este modo, la conservación y mejora de este grupo de especies en el olivar pudiera contribuir a una mejora en el control natural de ciertas plagas y a una menor dependencia de otros métodos de control tales como el uso de insecticidas de síntesis. En la presente tesis se han abordado varios aspectos del estudio de los crisópidos en relación al control biológico por conservación con el objeto de mejorar la presencia y diversidad de estos insectos en el olivar.

El Capítulo 4 describe una puesta a punto metodológica para experimentos de comportamiento de *Chrysoperla carnea s.l.* utilizando el sistema computerizado de seguimiento automático mediante video EthoVision XT. Esta metodología resultó adecuada para la identificación de distintos patrones comportamentales, correspondientes a diferentes conductas de búsqueda y alimentación, basándose en varios parámetros de movimiento calculados de forma precisa por el sistema EthoVision XT. La información proporcionada por estos ensayos indicó que esta metodología pudiera ser aplicada en experimentos de comportamiento de distinta índole. En el Capítulo 5, se investigó a diferentes niveles la compatibilidad del depredador *C. carnea s.l.* con un novedoso sistema de control de plagas, la aplicación de caolín, utilizado en el olivar para el tratamiento de la mosca del olivo. El caolín no produjo mortalidad directa a los estados preimaginales de *C. carnea s.l.* aunque el experimento realizado para la evaluación de la movilidad (utilizando la metodología descrita en el Capítulo 4) reveló que las larvas, al estar

cubiertas de una capa de caolín, mostraron cierta limitación en su locomoción, y que la capacidad de movimiento sobre superficie cubiertas con caolín también se vio afectada negativamente. Además, las larvas se desprendieron con facilidad de hojas de olivo tratadas con caolín lo que condujo a la hipótesis de que la reducción en la capacidad de movimiento y el desprendimiento de la superficie de la planta pudieran ser los principales efectos negativos del caolín sobre las larvas de *C. carnea s.l.* En cuanto a los adultos, las hembras de *C. carnea s.l.* mostraron una clara preferencia por hojas cubiertas con una capa de caolín para ovipositar. A pesar de ello, un experimento realizado a nivel de campo no demostró un incremento de adultos sobre árboles tratados.

Varios experimentos de campo fueron llevados a cabo en 10 olivares diferentes, y a lo largo de distintos años, con el objeto de establecer el efecto del uso de insecticidas y la presencia de cubierta vegetal (CV) sobre la abundancia y diversidad de crisópidos así como el efecto sobre estos enemigos naturales de los diversos tipos de manejo existentes en el olivar (Capítulos 6 y 7). Tres olivares con diferentes niveles de intensificación agrícola fueron muestreados en los años 1999 y 2000 y nueve olivares (tres por tipo de manejo; convencional, integrado y ecológico) en el año 2003. El uso del insecticida dimetoato no causó ningún efecto sobre larvas ni adultos de la especie más abundante, *C. carnea s.l.,* sin embargo afectó a *Dichochrysa* spp. produciendo un efecto negativo sobre sus poblaciones. Este hecho sugiere que las especies de la familia Chrysopidae pudieran mostrar una importante variabilidad en cuanto a su respuesta a insecticidas. También se concluyó a partir de estos experimentos que la intensificación agraria en el olivar está relacionada con una pérdida de diversidad de especies de la familia Chrysopidae contribuyendo a una mayor dominancia de la especie *C. carnea s.l.* Debido a la relación observada entre la ausencia de tratamientos herbicidas y el incremento en la presencia de adultos de *C. carnea s.l.* en los años 1999 y 2000, se evaluó el efecto de una CV espontánea separadamente del resto de factores de manejo agronómico durante los años 2009 y 2010. La CV, presente desde el principio de la primavera hasta el mes de junio, aumentó la abundancia de adultos de las dos especies más numerosas en los olivares andaluces *C. carnea s.l.* y *Dichochrysa prasina* (Burmeister), y

también contribuyó a un ligero incremento de la diversidad de especies. Un mayor número de adultos de *C. carnea s.l.*, debido a la presencia de la CV, se tradujo en un mayor número de larvas en la superficie del olivo coincidiendo temporalmente con la presencia de huevos de *P. oleae*. Por el contrario, las larvas de *Dichochrysa* spp. no incrementaron debido a la CV.

Finalmente, se desarrolló una técnica de marcaje, descrita en el Capítulo 8, para su uso en experimentos de marcaje-suelta-recaptura con el objetivo de estudiar el movimiento de crisópidos adultos para la mejora del control biológico por conservación en el olivar. Adultos de la especie *C. carnea s.l.* fueron marcados permanentemente induciéndoles un cambio de color, identificable principalmente en el abdomen, alimentándolos en estado de larva mediante el tinte soluble Sudan Red 7B incorporado a una dieta merídica. Se establecieron las concentraciones óptimas del tinte para garantizar un marcaje adecuado y una aceptable supervivencia de larvas y pupas sin causar ningún efecto sustancial sobre el desarrollo larval y su ingesta de alimento ni sobre la fecundidad y capacidad de vuelo de adultos.

1. General Introduction

1.1. Predators and biological control

The top trophic levels of terrestrial arthropods are natural enemies of herbivore insects contributing to a regulation of their populations in a particular agricultural ecosystem. This regulating effect was termed biological control and was early defined by Smith (1919) as the suppression of insects populations by the action of their native or introduced natural enemies. More recently, the scope of this definition has widened to include technological advances in the tools available for pest control. Thus, Van Driesche and Bellows (1996) defined this pest control method as "the use of parasitoid, predators, pathogen, antagonist or competitive populations to suppress a pest population making it less abundant and thus less damaging than it otherwise be". Biological control is generally regarded as the best possible pest management strategy on the basis of environmental considerations. Among the different options available entomophagous insects should be a primary consideration in pest management programs (Koul and Dhaliwal, 2003). The pest suppression capacity of entomophagous arthropods has largely remained unexploited due to the lack of knowledge and the underestimation of its potential (Hokkanen, 1993). However, predator and parasitoid application has gained increased attention in recent years due to the advantage provided by their ability to suppress the pest at the same time they reproduce at its expense, exerting a top-down effect on pest populations.

Predators are species that, at least in one life stage, kill and consume living animals for development, survival and reproduction. Predatory arthropods are widespread in the classes Insecta and Arachnida. The most prominent predatory insects belong to the orders Coleoptera, Neuroptera, Hymenoptera, Diptera, Hemiptera and Odonata (Van Driesche et al., 2008). Additionally there are a number of species of spiders and mites that prey upon a range of different pest species. Compared to parasitoids, predators are characterized by a high level of polyphagy and the generalized capacity to feed on more than one prey stage i.e. egg, larva (or nympha), pupa or adult.

Most of the predators regarded as beneficial insects, such as lacewings, coccinellid beetles and mites, show an agile and ferocious prey seeking behavior on the vegetation or at ground level. Some of them, as beetles, are equipped with chewing mouthparts for prey consumption while the sucking type of feeders (lacewings, bugs, hover flies) extract the internal body fluids of soft-bodied arthropods and eggs.

Insect's predatory activity was recognized and taken advantage for pest control long ago. There are accounts of ants used for citrus and date palm protection in the past and even the collection of ladybirds for release against pest in the 19th century (Orr and Suh, 2000). To date theoretical and experimental approaches have concentrated mostly on biocontrol carried out by specialist, and almost exclusively, parasitoids. Nonetheless, it has been widely acknowledge that assemblages or guilds of predators can perform as effective biological control agents against populations of both indigenous and exotic pests. Symondson et al. (2002) observed that generalist predators were able to significantly reduce pest infestation in about three-quarters of the field experiments reviewed. In fact, despite polyphagy entails important drawbacks for biological control performance (the reduction of functional response in presence of alternative prey, for instance) it is currently believed that in practice, there are trade-offs between the potential of specialist biocontrol agents and generalists predators. Thus, predator populations may be sustained in acceptable numbers when prey densities are low by subsisting on non-pest species in what has been referred to as a lying-in-wait strategy (Symondson et al., 2002). This ecological characteristic confers them the ability to act efficiently right from the beginning of the pest attack.

1.2. Conservation biological control

Biological control methods are widely accepted to be based on three major approaches: Introduction, augmentation and conservation. Introduction also referred to as importation or classical biological control (due to its historic predominance) is based on the establishment of an imported natural enemy of proven effectiveness in the suppression of the target pest. Augmentation

methods aim to provide the agricultural ecosystem a sufficient numbers of the biological control agent that is naturally scarce or not present at the right time. These methods are generally characterized by mass culture of insects and periodic releases. Finally, conservation biological control can be defined as the optimization of the modifications of human influence with the objective of increasing the extent to which natural enemies are able to suppress pests (Orr and Suh, 2000; Van Driesche and Bellows, 1996). Augmentation and conservation biological control can be considered compatible and even complementary approaches and have been regarded as the two extremes of a continuum, one extreme consisting of rationalizing agricultural practices in order not to reduce naturally occurring populations (conservation) and the opposite consisting on natural enemy mass release (augmentation). The halfway point in this continuum would be occupied by the environmental modifications aiming to both preserve and increase natural enemies, traditionally under the category of conservation (Ehler, 1998). Conservation biological control has been a rather disregarded practice compared to the more conventional introduction and augmentation methods. However, research in this area is progressively gaining importance in the last decades (Gurr et al., 2004; Wade et al., 2008a; Zehnder et al., 2007) as it can contribute to safer biological control, acceptable and adoptable by farmers and suitable to support environmentally friendly marketing strategies (Jonsson et al., 2008). Many authors believe that conservation biological control should be considered the keystone to biological control application (Gurr and Wratten, 1999).

Eilenberg et al. (2001) grouped within the category of conservation biological control all the methods utilizing pre-existing biological control agents in a given agricultural ecosystems. They thus identified two aspects of conservation biological control; the protection of natural enemies, and the provision of adequate resources to improve their numbers, fitness and effectiveness in pest control. Protection is clearly related to the limited and selective use of pesticides and other pest control strategies entailing non-target effects to biological control agents. Food and shelter resources may be provided directly or by adjacent or within crops habitats manipulation (Landis et al., 2000).

1.2.1. Insecticidal methods and natural enemy conservation

Agricultural ecosystems are in essence disturbed ecosystems with a decreased presence of natural enemies derived from the loss and fragmentation of their natural habitats during the land-use conversion process. However, once established the new ecosystem there are other factors that potentially affect natural enemies abundance and diversity. New (2005) identified agricultural intensification as a major contributor to arthropods biodiversity loss in agricultural ecosystems. Intensification is inevitably coupled with the necessity of crop protection by using pest control strategies that involve predominantly the elimination of insects. These organisms are closely related to natural enemies, coexisting in the very environment were the targeted pests are present. Crop protection strategies disregarding the interaction between pest and natural enemy complexes may lead to undesirable consequences such as a reduction of the ecological service they provided in controlling the targeted pest, secondary pests outbreaks and the development of pesticides resistance (Ruberson et al., 1998). The integrated pest management (IPM) approach was born with the philosophy of combining the use of natural enemies and the most extended pest control methods, the chemical control (Stern, 1959). Later on, the concept was extended to include other viable measures as crop resistance and cultural aspects. However, the key continues to rely on the possibility of integrating insecticidal methods and conservation biocontrol in comprehensive pest management programs, including not only pesticides applications but also other methods that may be hazardous for natural enemies such as biopesticides or mass-trapping. An improvement on the knowledge of the interactions between insecticidal methods with the most valuable natural enemies in a given crop is essential to increase their pest suppression compatibility.

There are several means for achieving this integration. Detailed phenological data on the natural enemies and pests trophic relationships may allow for a correct application timing causing minimum disturbance to their beneficial action as shown by several studies (Murchie et al., 1997; Wilson et al., 1998). Spatial separation from natural enemies such as spot treatments,

that restricts applications to specific plant parts, the use bait formulations, or a combination of these practices, may also reduce the damage caused to natural enemies. However, without doubt, the most powerful means for the achievement of natural enemies compatibility is the use of selective insecticides (Hull and Beers, 1985) and selective insecticidal methods in general.

The use of insecticidal methods may reduce natural enemy effectiveness due to several negative outcomes. Most pesticides of common use are directly toxic to natural enemies depleting their populations (Bartlett, 1963). It is also reasonable to assume that any kind of insecticidal compound, including soaps, oils, kaolin or fungal and microbial bioinsecticides may also cause mortality to natural enemies (Hassan, 1989). Besides the increased mortality, some of these compounds may also alter natural enemies' biological potential to suppress pests due to sublethal effects. These effects can be classified in physiological (reduced longevity and oviposition, longer development rates, altered sex ratio) and behavioral (alterations in foraging capacity, oviposition or behavior), and as lethal effects, depend on each natural enemy physiology and ecology (Van Driesche et al., 2008). Appropriate measurements of the impact of insecticidal methods on all the above listed parameters are necessary in order to determine their selectivity, and therefore, their compatibility with key natural enemies.

1.2.2. Habitat management

Conservation biological control seeks to favor natural enemy wellbeing in an attempt to increase their natural pest control efficiency through the manipulation of the agricultural ecosystem. As we have seen before, human influences may be adapted to minimize negative impacts but can also contribute to improve the availability of the resources necessary for natural enemy optimal performance. These manipulations may involve practically any aspect concerning the provision of supplementary or essential resources (New, 2005). Agricultural ecosystems, depending on the level of management intensification, may be hostile environments for particular natural enemies

limiting their effectiveness. Conservation biological control practices intend to partially restore those missing resources promoting the normal development of parasitoids and predators ecological function. The possible alternatives are indeed wide. The action of natural enemies can be enhanced by facilitating the interaction on the crop surface. There are crop varieties more appropriate than others due to traits such as favorable plant surface, non-toxic plant tissues and more nutritive flowers (Ode, 2006). Soil management must also be taken into account as practices, such as tillage and mulching, impact the environment of soil-dwelling natural enemies affecting their performance. The direct provision of food, both as attractant and arrestant of foraging natural enemies, has been successfully employed (Wade et al., 2008b) and the spray of previously tested chemical attractants to increase locally natural enemies populations is a promising option yet to be exploited (Koczor et al., 2010; Tóth et al., 2009).

Nevertheless, most of the research efforts to conduct conservation biological control have been devoted to the role of vegetation biodiversity (Andow, 1991). The enemies hypothesis holds that the effectiveness of predators and parasitoids increases in relation to the plant diversity in a given environment (Russell, 1989). This hypothesis is based on the fact that diverse agroecosystems, as opposed to monocultures in the sense of Andow (1991), ensure higher level of resources availability. Plant diversity may be improved in a number of ways within an agricultural management scheme by the implementation or maintenance of non-crop vegetation or polycultural practices (Fig. 1.1).

Non-crop vegetation, within and around farms, or farmscaping components, as viewed by Pickett and Bugg (1998) provide refuge, overwintering shelter, food resources of vegetal origins and alternative prey when not present in the crop. Predators and parasitoids take advantage of these resources feeding, reproducing and subsisting, and at the precise moment spill-out into the crop plants to act over pest outbreaks. The use of these resources for pest control purposes is termed habitat management and was defined by Fiedler et al. (2008) as “the intentional provision of flowering plants and plant communities in managed landscapes to enhance natural enemies”. The beneficial effects of structures as field margins, hedgerows and

cover crops on natural enemy abundance have been addressed by several authors (Bianchi et al., 2006; Silva et al., 2010) and there is evidence that it can translate in a more efficient pest reduction (Skirvin et al., 2011). Nonetheless, Landis et al. (2000) noted that in order to enhance natural enemies, the "rigth" plant diversity should be provided arranged spatially and temporally in a way they can contribute to pest suppression. In this sense recent research has focused on plant selection according to predators and parasitoids needs (Fiedler and Landis, 2007). In the particular case of predators, habitats with a great variety of prey and other food resources may be more critical than to parasitoids due to their mentioned polyphagous habits (Barbosa and Wratten, 1998).

According to its definition, habitat management has also a component at scales above farm or orchard. Due to practical reasons, related mostly to agricultural management, habitat establishment and manipulation primarily relies in local improvements at farm or orchard scale. However, diversified landscapes with an increase in elements such as woody vegetation and remnant patches of non-crop land have been linked to higher abundances of predators and parasitoids (Bianchi et al., 2006; Thies and Tscharntke, 1999). It is believed that structurally complex landscapes compensate for the disturbance produced locally by intensive farming due to the re-colonization capacity of highly dispersing natural enemies (Tscharntke et al., 2005). For example, lacewings, known to be nomad predators in agricultural ecosystems, have been observed to show higher species richness in agricultural fields in relation to the amount, type and density of non-crop patches of the surroundings (Szentkirályi, 2001a). Future research addressing landscape influence on predators and parasitoids might help to include landscape design to the habitat management "toolbox", or at least, to make reliable recommendations at landscape level.

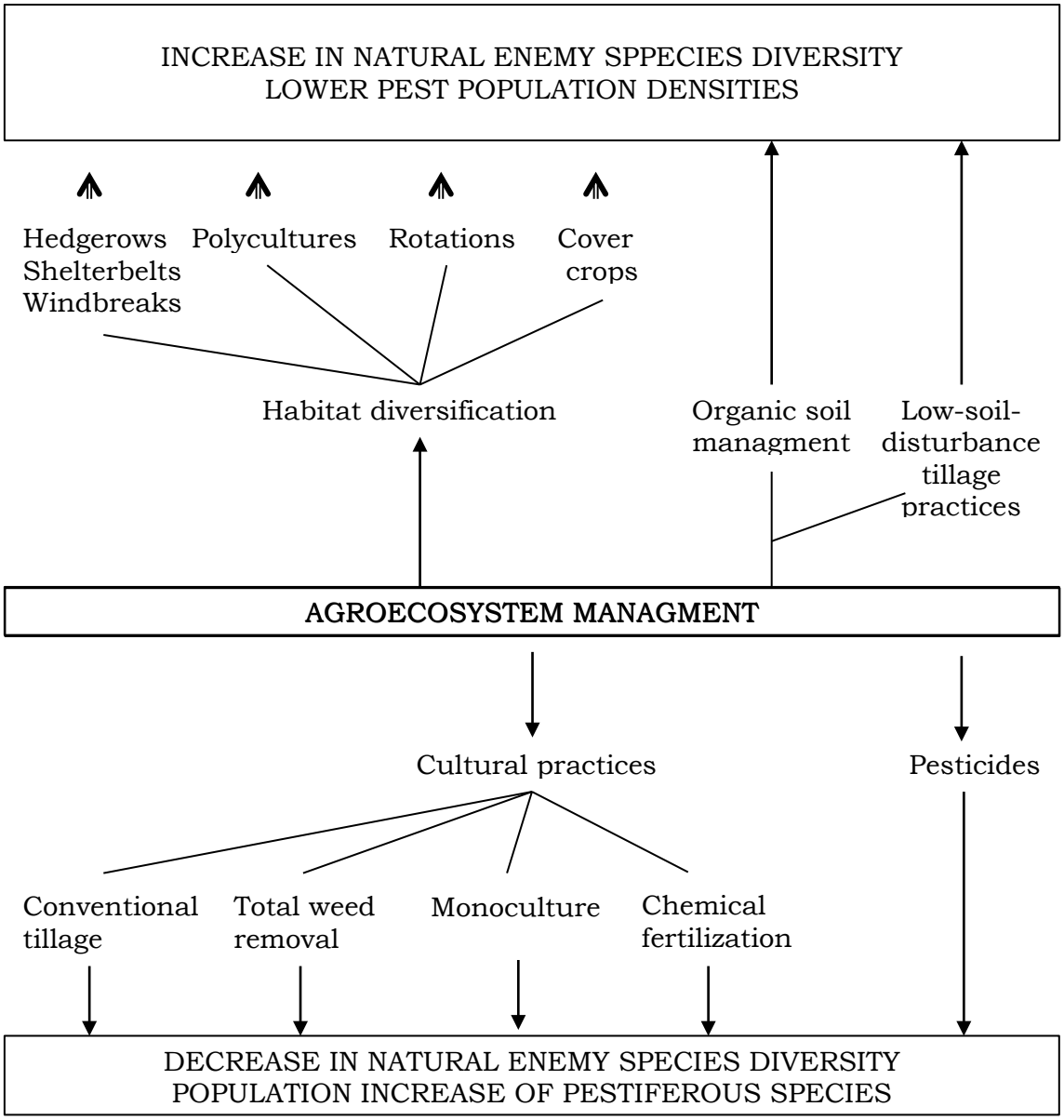

Figure 1.1 Effect of management practices on biodiversity of natural enemies and abundance of insect pests (Altieri and Nicholls, 1998).

1.3. The family Chrysopidae

1.3.1. Taxonomic status

The family Chrysopidae Schneider, 1851, belongs to the order Neuroptera *sensus stricto*, also known as Plannipenia, a reduced group of species among the most primitive of the holometabolous insects. This order is classified within the superorder Neuropteriodea along with the orders Megaloptera and Raphidioptera, group also referred to as Neuroptera *sensus*

lato. Neuroptera adults are generally characterized by a complex wing venation, which gives them their name, with numerous crossveins and often lacking pigmentation. The order includes about 6000 species that belong, up to now, to 18 different families mostly terrestrial with a few exceptions as Sisyridae (New, 2001). Adults and larvae are distinct in appearance and mostly of predatory habits. Common recognizable characters of larvae such as fused mandible and maxilla into a suction tube, and a closure between mid and hindgut, show overwhelming evidence of a common monophyletic origin of the group (Schluter, 1984). There are three families of Neuroptera sufficiently abundant in agricultural ecosystems to be considered of economic interest for biological control, the family Coniopterygidae, or dusty-wings, Hemerobidae, the brown lacewings and the most relevant, the family Chrysopidae (Stelzl and Devetak, 1999).

The Chrysopidae or green lacewings are the most common and diverse group of species among the order Neuroptera. It is a relatively large family comprising 75 valid genera and incorporating some 1200 specific names worldwide according to the latest revision on the family systematics (Brooks and Barnard, 1990). Stelzl and Devetak (1999) defined adults as medium or large insects mostly greenish in appearance, and with fore wings about 6–35 mm long (Fig. 1.2a) and their offspring as campodeiform larvae, upto 2 cm long, and with visible suctional jaws (like all Neuroptera) curved towards each other (Fig. 1.3). Monserrat (2001) described several distinctive features compared to the rest of neuropteran larvae some of the most relevant being large head, jaws and antennae, conspicous setigerous tubercles on both thorax and abdomen, and empodia between tarsal claws. Again, according to Brook and Barnard (1990) adult chrysopids are divided in three different subfamilies: Nothochrysinae, Apochrysinae and Chrysopinae. Nothochrysinae are regarded as the most

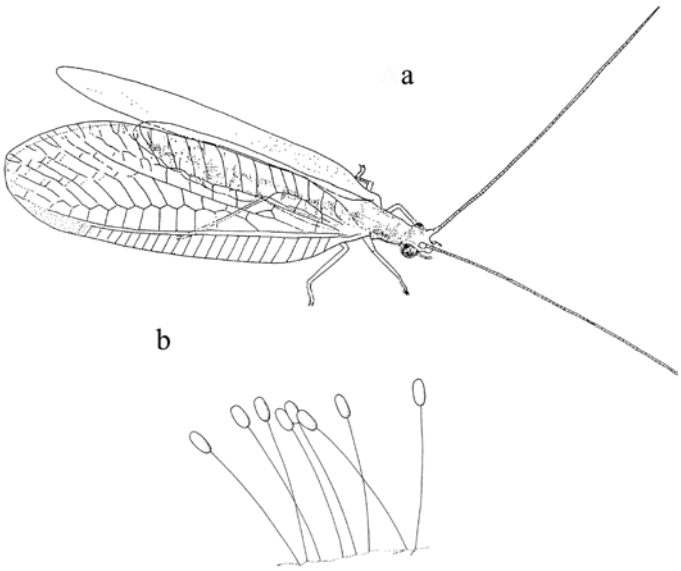

Figure 1.2. General aspect of Chrysopidae a) adult and b) eggs (Stelzl and Devetak, 1999).

primitive group of Chrysopidae as well as the least diverse with only less than 20 species split in nine genera. Adults are relatively big and lack the alar tympanic organ present in the rest of subfamilies. Only three species are present in Europe. Apochrysinae are ussually large and exhuberant individuals with dense venation and associated to tropical forests. It is also a very small group with no species present in Europe. Finally, the great majority of species of the family (97%) belong to the vast and widespread subfamily Chrysopinae, comprised by four different tribes: Anlylopterygini, Belonopterygini, Leucochysini and Chrysopini. Among them, the tribe Chrysopini stands out as the most numerous with 30 different genera recently separated in seven different sub-clades by a molecular phylogenetic survey (Haruyama et al., 2008).This group includes virtually almost all the species of agricultural relevance such as those belonging to the genera *Chrysoperla*, *Dichochrysa* and *Chrysopa* (New, 2001).

1.3.2. Outlines of life history and behavior

Green lacewings biology, and particularly that of the species of economic and applied interest, is well-known and has been reviewed recurrently in the literature of the field (Canard et al., 1984; Canard and Volkovich, 2001). Female chrysopids lay eggs singly or in cluster depending on the specie, mostly on leaves, preferring the underside for oviposition, with a minor amount laid on branches or on the tree trunk (Szentkirályi, 2001a). Lacewing eggs are oval, up to 3 mm in length, and may be white, yellowish, bluish or green, also characteristic of the species (Fig. 1.2b) (Monserrat et al., 2001). With the exception of the genus *Anomalochrysa*, all the green lacewings lay their eggs at the tip of a silken stalk which is attached to the substrate at the other end. This characteristic trait has been long assumed to provide the egg with protection against other predatory arthropods such as ants and coccinellid beetles and to avoid cannibalism (Ruzicka, 1997).

After hatching has taken place the newly emerged predatory larva initiates an active search for prey with activity increasing as a function of starvation (Sengonca et al., 1995). The prey encounter happens at random, as a consequence of the search activity. The larva handles the prey by quickly catching it and subsequently sucks up its internal tissues (Canard, 2001). Chrysopid larvae are polyphagous predators known to prey on a wide range of small, soft-bodied insects, spiders and mites as well as on eggs and lepidopteran larvae (Principi and Canard, 1984). According to morphology and behavioral patterns there are two types of chrysopid larvae (Gepp, 1984; Monserrat et al., 2001). The trash-carrying type of larvae (e.g. the genus *Dichochrysa*) gather prey remains and plant material on their dorsum using it as a protective camouflage against their antagonists. The nude type (e.g. *Chrysoperla*) lacks this habit but are, in general quicker, and more active. Preimaginal development is highly similar across the family undergoing three larval stages and two molting events. The third and last larval instar, once it has reached full growth, goes through a short period of reduced food intake after which spins a subspherical cocoon using a silk secretion from the Malpighian tubules. The choice for a pupation place differs greatly among species. Some abundant chrysopids in agroecosystems, such as *Chrysoperla carnea s.l.* and *Dichochrysa prasina* (Burmeister), spin their cocoon in relatively open locations on their host plant as the underside or even the upper side of the leaves. Other species, however, move towards the ground and prefer more concealed shelters as litter or the soil at a shallow depth (Canard and Volkovich, 2001). The larva stays inactive inside the cocoon, known as the prepupa state, and eventually molts to pupa squeezing the molt remains against the cocoon walls making them clearly visible from outside as

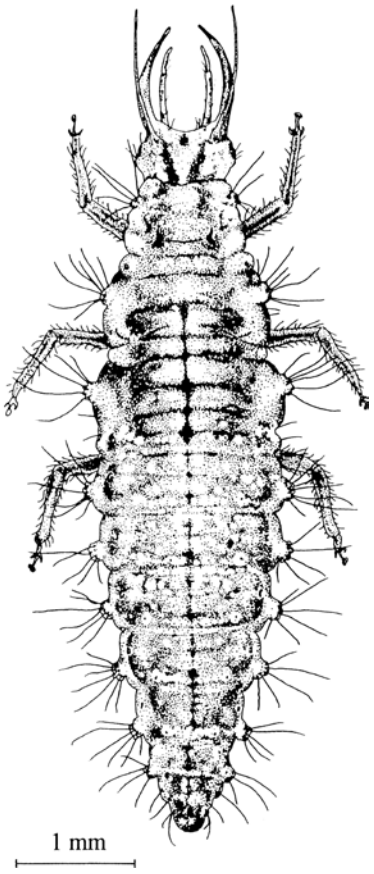

Figure 1.3. The third instar larva of *C. carnea s.l.* (Gepp, 1989)

a black dot. The end of the preimaginal period constitutes a critical time in development of green lacewings. The decticous pupa or pharate adult breaks out of the cocoon with its mandibles and, in this torpid and vulnerable state, seeks an adequate vertical substrate to finally molt to imago.

Soon after emergence, species of the genus *Chrysoperla* have been reported to carry out preovipository migration flights for two consecutive nights (Duelli, 1980; 2001). Flights are continues, follow the wind direction and are characterized by the lack of response to food sources and mating stimuli. After this period, in the case of some *Chrysoperla* spp., and after emergence in the case of other chrysopids, adults begin a foraging and mating activity known to happen mainly after sunset (Szentkirályi, 2001a). As adults, some species are able to feed on the same prey as their larvae i.e. aphids, and other soft-bodied arthropods, however, their feeding habits also include pollen of different sources and they thereby have been classified as omnivorous (e.i. *Chrysopa* spp.) (Stelzl and Devetak, 1999). Nonetheless, most of the species feed on vegetal substances such as pollen, nectar and other vegetal exudates, and honeydew secreted from aphids and other insects, in what is regarded as a palyno-glycophagous regime (Canard, 2001). Hence, adult Chrysopidae are believed to be specially attracted to patches of flowering plants (Villenave et al., 2006) and artificial floral baits have been observed to exert an arrestment effect towards *Chrysoperla* spp. (Koczor et al., 2010; Tóth et al., 2009).

The sexual behavior of chrysopids has been investigated in detail for some representative species although little is known about some important genera. All the species observed show a highly characteristic courtship communication during the mating process described by Henry (1984) as "complex substrate-borne vibrations produced by vigorous, stereotyped jerking motions of the insects' abdomen". This sexual behavior has important implications on chrysopids specification that are addressed individually elsewhere within this introductory section. Some species of the genus *Chrysoperla*, shortly after the cessation of the obligatory migration flight triggered by a starting response to food stimuli, are ready to duet, mate and reproduce. However, unlike other insects with similar adaptative migration flights, these *Chrysoperla* spp. continue dispersing during their reproductively active period in an strategy that has been regarded as an adaptation that

provides a higher foraging success in patchy environments such as agricultural fields (Duelli, 2001). Even though this behavior has only been observed in two species, Duelli (2001) suggested that similar migrating strategies might be common in chrysopids that are ecologically succesful in agricultural ecosystems.

Concerning voltinism, despite the variability observed throughtout the different species of the family, multivoltinims is the most usual seasonal succession of generations in green lacewings (Canard and Volkovich, 2001). True univoltine species, as *Rexa lordina* (Monserrat, 2008), are rarer, and a sole generation is usually linked to local environmental conditions in species that otherwise would be moltivoltine. Multivoltism leads to generational overlaps and therefore it is quite common for certain species the coexistence of all the development stages during high activity seasons. Diapause is responsible for green lacewings generational cycle regulation, and with the exception of embryonic diapause, all forms have been recorded in the family Chrysopidae, being larvae and adult diapause the most commom (Principi, 1991). In agricultural ecosystems, crop especialists lacewings are belived to carry out flights to and from overwinterings sites apart from the other type of flights described above (Duelli, 2001).

1.3.3. Chrysopids and agriculture

One of the differential characteristic of agricultural ecosystems vs. natural ecosystems is the imposed temporal and spatial uniformity of plants that determines a reduced vegetal diversity (Pedigo, 1996). A simplified and ecologically unbalanced ecosystem is one of the main reasons underlying pest outbreaks (Altieri and Nicholls, 2004). In this sense, the resource concentration hypothesis links large homogeneous patches of host plants to an increase in phytophagous populations as an explanation for agricultural pests (Root, 1973). As noted above, especial adaptations of some Chrysopidae species to difficult environments has facilitated their establishment in agricultural ecosystems, and furthermore, have benefit their dispersion by extending their distribution range alongside the increase in land use for agriculture (Duelli, 2001). Thus, green lacewings are present in almost all the agricultural ecosystems around the world consuming and reproducing on

phytophagous pest insect associated to each crop, being, for instance, among the best known and active aphidophagous predators.

Stelzl and Devetak (1999) identified the species *C. carnea s. l.*, *Chrysopa phyllochroma* (Wesmael), *Chrysopa. formosa* Brauer, *Chysopa. pallens* (Rambur), *D. prasina* and *Dichochrysa flavifrons* (Brauer) (in Mediterranean crops alone) as the frequently encountered in agricultural sites, and thereby the best adapted to the agricultural environment. However, Chrysopidae assemblages in different crops usually record higher richness values (Szentkirályi, 2001b; Szentkirályi, 2001c) which can be related to reinvasion from natural or semi-natural surrounding areas (Stelzl and Devetak, 1999) or to an specificity towards a given substrate mediated by the existence of an specific phytophage on the plant or a vegetal substance on which chrysopids feed (Monserrat and Marin, 2001). In field and vegetable crops, attacked by many pests including numerous aphid species, the knowledge regarding chrysopids is sparse despite they are quite common among the natural enemy guild (Szentkirályi, 2001c). Much more is known on their activity in orchard crops, extensively reviewed by Szentkirályi (2001b), which constitute more stable agricultural ecosystems than field crops. As an example, green lacewings species exert a natural control on *Aphis pomi* De Geer populations in apple, the pear psyllid (*Cacopsylla* spp.) in pear orchards, different aphid species in peach and cherry crops, mites and leafhoppers in grape and several aphids in citrus. Chrysopids, in accordance to their polyphagous predator condition, have often been also observed feeding in other pests of these crops such as scales, mealybugs and lepidoteran eggs. Due to their cosmopolitan distribution, the list of crop fields where chrysopids have been found to attack insects pest include tropical and subtropical orchards, nuts crops, sugar crops, berry crops and ornamental plants.

Green lacewings have been long recognized to fulfill most of the requirements to be an effective biological control agent (Senior and McEwen, 2001). *Chrysoperla* spp. prey upon an important range of pests and are efficient predators, mass-rearing is manageable and development quick, and their action is compatible with several pest management strategies (Pappas et al., 2011). In addition, their worldwide distribution makes them candidates to be used in almost any possible agricultural ecosystem (New, 1984).

Consequently, many insectaries worldwide rear and commercialize insects of the genus *Chrysoperla* for mass release into the crops as part of an augmentation strategy (Henry and Wells, 2007). In orchards, *C. carnea s.l.* has been released to successfully control citrus pests, leafhoppers in vineyards, mealy bugs in pear orchards, and *A. pomi* in apple orchards (Szentkirályi, 2001b). Species of the genus *Chrysoperla* have been also released to control *Helicoverpa zea* (Boddie), the European red mite, the Colorado potato beetle and many aphids mostly in greenhouse (Rigway and Murphy, 1984; Tulisalo, 1984). The vast majority of research on the evaluation of augmentative biological control based on the use of green lacewings has been devoted to the *C. carnea s.l.* group of species. However, species of the genera *Mallada* and *Dichochrysa* and the species *Ceraeochrysa cubana* (Hagen) are currently candidates for mass production (Pappas et al., 2011) and recent studies have shown at laboratory level that the cosmopolitan species *D. prasina* might be an adequate option (Pappas et al., 2007; 2008). Nonetheless, to date economic feasibility remains the limiting factor for augmentative release of chrysopids in agricultural fields. The labor intensive mass rearing procedure is still a handicap for an industry that requires further research into the matter (Senior and McEwen, 2001).

1.3.4. The sibling species of the carnea-group

As mentioned before, the 36 recognized species of the genus *Chrysoperla* have traditionally been the most relevant Chrysopidae in biological control programs (Brooks, 1994) as well as, in general, the most abundant naturally occurring species of the family in agricultural ecosystems (Szentkirályi, 2001b). The most frequent chrysopid natural enemy referred to in the literature is the species *Chrysoperla carnea* (Stephens), believed in the past to be a single species extended to nearly all the northern hemisphere, and to which a lot of names had been incorrectly assigned due to the numerous subspecies or varieties existent (Canard and Thierry, 2007). The extremely high morphological homogeneity of these populations kept this group under the same name for long. It was not until the beginning of the pioneer works by Henry (1979) on the different substrate-borne vibrations produced by males and females during premating courtship, that robust evidence for species identification was provided. Henry (2001) situated differences among this

courtship songs at the core of a dynamic speciation process determined by reproductive isolation. Thus, he was able to categorize up to 12 unique song phenotypes in North America and Europe corresponding each one of it to a different song species of the so-called sibling species of the *carnea*-group. In Western Europe there are five recognized courtship song species; the originally termed *C.c.2* to *C.c.4* and the morphologically distinguishable species *Chrysoperla. mediterranea* (Holzel) and *Chrysoperla lucasina* (Lacroix) (Henry, 2001). In Mediterranean countries the problem is reduced to just *C.c.2* and *C.c.3*, as *C.c.4* has not been collected and identified through its characteristic song (Henry et al., 2002). During the last years there has been certain taxonomic debate among authors on the correct denomination of some of the species resulting in a current lack of nomenclature consensus among specialists (Canard and Thierry, 2007).

Courtship songs analysis constitutes sufficient evidence of pre-mating reproductive isolation, however, the fact that these species can produce non-sterile hybrid offspring (weak post-mating species isolation) and the low genetic divergence among them are indicatives of the a very recent and rapid speciation process (Lourenço et al., 2006; Noh and Henry, 2010). Differences in distribution seem quite clear and there is certain evidence for ecological differences in vegetation strata preferences among species (Duelli, 2001; Henry et al., 2002; Henry et al., 2003). However, environmental adaptation to different ecological niche does not seem to be influencing this speciation process as vegetation strata does not affect courtship songs in any way, and there are examples of song species able to adapt to different strata depending on the site they live in (Henry and Wells, 2004; Thierry et al., 2011).

At a practical level, Henry (2001) himself recognized the limitations encountered by entomologists when addressing the sibling species problem. In order to identify them reliably, non-diapausing adults must be collected alive and specific equipment and training are required. Morphological characteristics can be used for distinguishing certain cryptic species locally (Chapman et al., 2006), but these features are mostly ambiguous and differing among sites, influenced even by the different species living in sympatry (Thierry et al., 2011). Some of these evident drawbacks result in no possible solution to the sibling species problem satisfactory for all specialists (Henry,

2001). Some authors doubt the species status of this group regarding them as biotypes of the same species with a high degree of variability (Tauber and Tauber, 1987; Tauber et al., 1997). In applied entomology, while it is still quite common for authors to refer to the specimens collected in the field as *C. carnea* (Corrales and Campos, 2004; Pathan et al., 2010), it seems advisable the denomination of *C. carnea sensus lato* in order to acknowledge this diversity of species when no further identification is possible (Canard et al., 2007; Koczor et al., 2010).

1.4. The olive agricultural ecosystem

1.4.1. The olive crop

The olive tree (*Oleae europaea* L.) has been cultivated for olive oil production, olive fruits and its wood from the days of early civilization. There is evidence that olives stand among the first cultivated fruit trees initiating probably in the copper age between 5700 and 5500 years ago in the modern Jordan. Its expansion throughout the Mediterranean basin began in the first millennium B.C. with the increasing activity of Phoenician and Greek merchant civilizations and with Roman domination (Pajarón-Sotomayor, 2008). Nowadays, the total olive trees worldwide have been estimated in around a 1000 million extended in an area of 10 million hectares that has been incrementing annually from 2001. The 98% of this total is concentrated in the Mediterranean region where it is regarded as an emblematic crop, however, in recent years the crop has expanded to other continents such as America with 1.2% of the production, Asia (0.4%) and Australia and Oceania (0.4%). Olive oil commerce and production is led by the EU that produces the 78% of the world's total olive oil and is the major exporter and importer from other Mediterranean countries (Civantos, 2008). Among the countries, Spain is the first olive producer in the world (39%) with olive orchards present to some extent in almost the complete national territory. The total agricultural surface dedicated to this crop in Spain was reported to be 2.572.793 ha in the year 2010 (ESYRCE) showing an increasing trend from 1984 (Civantos, 2008). The increase in olive oil production can be partly explained by the growing volume of exportation to non-producer countries within the European Union and to other countries such as USA and Japan that has occurred during the last ten

years. Factors as the well-defined olive oil health benefits and its innovative use for manufacturing other industrial products have favored this growing market.

In Andalusia, olive cropping accounts for 32% of the worldwide olive oil production and a 3% of the gross domestic product of the region. Apart from this economic importance it also plays a relevant role for social and territorial cohesion and is relevant from a cultural point of view. Indeed, many regard this crop as a representative landscape feature part of the Andalusian identity as it represents the highest concentration of a cultivated arboreal species in all Europe spread along nearly 300 km from northeast in the province of Jaén to southwest in Cádiz (Guzmán-Álvarez et al., 2009). Production systems may vary extraordinarily throughout the region ranging from highly intensive orchards in non-traditional olive growing areas to traditional low-productive orchards situated in uplands and highlands (Rubio-Pérez et al., 2002). This high variability has its origins in the different socioeconomic realities throughout recent history that have affected the evolution in the management of this crop (Guzmán-Álvarez et al., 2009). The second half of the 20th century witnessed a transition from the traditional management methods to modern agriculture heavily dependent on external outputs for irrigation, fertilization and chemical control of pests and diseases. The new model achieved excellent production standards but largely neglected other aspects as the social dimension of the activity, and mainly, the environmental impacts of this practices resulting in negative effects such as soil erosion and contamination and biodiversity loss (Gómez et al., 2009; Ruano et al., 2004). Aware of this situation, there is an increasing social demand for olive oil and table olives produced under more sustainable management conditions, with a consumers desire to access residue-free products and to keep the environment free from agrochemicals (Vossen, 2007). Furthermore, the European agricultural policy has been changing its focus and is expected to continue in the future taking a marked “green” turn towards sustainable exploitation of agricultural resources and climate change mitigation measures. From 2014 to 2020 producers will be encouraged through economic mechanisms to protect the environment and benefit biodiversity within agricultural ecosystems. This has led Andalusian local authorities to developed policies favoring integrated and ecological olive management systems in the detriment of the management conventionally used

in the recent past. Integrated and organic management systems are certified by the administration and have their own individual characteristics whilst the conventional management is defined by exclusion from the other categories.

Organic agriculture is based on principles of sustainable development and is defined as a management system with the objective of obtaining maximum quality products, conserving soil fertility as well as the rest of the environment, through the optimal use of the natural resources (Garrido et al., 2009). This system excludes the use of any kind of synthetically synthesized agrochemicals. Andalusian organic agriculture certification is common to all the agricultural products and covers all the production processes from agricultural inputs to commercialization and exportation. Organic olive production started in the 80' and reached in the year 2008 the extension of 41,596 ha (Garrido et al., 2009). This cultivating system has been observed to provide several advantages over the rest. The product quality is increased, and, from an environmental point of view, it achieves higher levels of soil protection and biodiversity and lower levels of soil and water pollution (Alonso and Guzmán, 2006; Pajarón-Sotomayor, 2008).

Integrated production, pioneered by the Junta de Andalucía, can be defined as an agricultural system seeking to optimize production mechanism and resources with the objective of a long-lasting sustainable agriculture. This management system allows for biological and chemical control methods and other practices aiming to make compatible environmental protection and agricultural production (Garrido et al., 2009). Local authorities developed a specific regulation for integrated olive production in 1997 that has been revised and improved periodically. This production system is characterized by a high technical support for management decision making provided by the administration, along with a control for the correct application of the measures covered by the regulation.

Integrated production deals with pests and diseases problems under a sustainable point of view integrating all the possible options with special emphasize on natural regulation of pest populations. In this context, chemical insecticide use, although allowed, is regarded as the last option among those available and only applicable under certain infestation circumstances when

alternative measures prove to be insufficient. The organic agriculture measures for pest management are very similar with a primary focus on prevention through cultural methods and natural regulation of phyophages populations. The main difference is that no chemical insecticides are allowed and may be substituted by natural products with insecticidal properties when required. Conventional management methods are very varied, however, they rely in chemical control as the main pest management strategy. Unjustified systematic applications for controlling the main olive pest insects are not unusual.

1.4.2. The entomological fauna of the olive agricultural ecosystem

The olive agricultural ecosystem is rich and diverse in arthropod species. There are about a hundred phytophages, nearly a thousand predators and parasitoids, several pollinators and a great group of insects regarded to be neutral in their interaction with the crop plant (20%) (Arambourg, 1986; Campos and Civantos, 2001). Neutral species, despite their indirect implication with the crop, have been noted to play an important role in agricultural ecosystems by decomposing organic matter or allowing the presence of natural enemies acting as an alternative host or prey (Nicholls et al., 2001).

Almost the complete range of phytophage insect orders are present in olive agricultural ecosystems but just a reduced part of this diversity are relevant in terms of economic loss (Quesada-Moraga et al., 2009). The olive fruit fly, *Bactrocera oleae* (Gmelin), is largely regarded as one of the, or even the most, severe pests in olive cropping. Adults oviposit on nearly full-grown olive fruits and, after the eggs have hatched, the larva consumes part of the pulp making the fruit fall and useless for commercialization as table olives and affecting drastically the olive oil quality resulting from attacked olives (Civantos, 1999). Another major pest that attacks directly the olive fruits in *Prays oleae* (Bernard) also known as the olive moth. *P. oleae* has three generations showing a remarkable synchrony with the host plant phenology. The leaf or phylophagous generation develops to adulthood consuming the inner side of the leaves. The next generation, called the anthophagous, feeds on the flower bud devouring anthers, stigma and ovaries. Finally, the flower

generation adults mate and oviposit on the small fruit, mostly on the remains of the calyx near the peduncle. Third generation or carpophagous larvae penetrate the fruit, feed from the inside, and around two months later exit the fruit to pupate triggering the fruit fall on their way out (Alvarado et al., 2008). The black scale *Saissetia oleae* (Olivier) is considered a pest that produces harm to the crop under certain circumstances such as other homopteran, the psyllid *Euphyllura olivina* (Costa). There are numerous minor or secondary pests that can cause local crop damage as the pyralid *Euzophera Pingüis* (Haworth) and the bark beetles *Phloeotribus scarabaeoides* (Bernard) and *Hylesinus oleiperda* Fabricius.

The parasitoids present in olive orchards have been estimated in around 300 or 400 different species belonging to the order Hymenoptera. Some of the most relevant species for biological control purposes are: *Eupelmus urozonus* Dalman attacking the olive fly, *Trichogramma* spp., *Ageniaspis fuscicollis* (Dalman) and *Chelonus* spp. attacking the olive moth, and *Metaphycus* spp. and *Scutellista caerulea* (Fonscolombe) attacking the black scale (Arambourg, 1986). The predators in olive orchards are represented by different orders. Spiders and ants are the most abundant and diverse (Cárdenas et al., 2006; Morris et al., 1999a; Redolfi et al., 1999). The coccinellid community is also significant (Cotes et al., 2010). However, other predators which are also present and that possess a different feeding pattern, such as anthocorids and chrysopids, are believed to play important roles in phytophages natural control in olive trees (Campos, 2001; Pantaleoni et al., 2001).

Thus, the olive agricultural ecosystem is regarded as quite an ecologically stable ecosystem due to the relatively scarce number of harmful pest insect and the abundance and diversity of their antagonists that contribute to their natural suppression (Cirio, 1997). This network of ecological relationship is thereby worth conserving and promoting in order to avoid pest outbreaks by applying principles and measures of conservation biological control.

1.5. Green lacewings in olive orchards

Olive orchards are among the most investigated agricultural ecosystems regarding the presence and predatory activity of green lacewings as well as the crop for which higher green lacewings species richness has been detected (Szentkirályi, 2001b). This fact may be explained mainly by the relevance for biological control attributed to chrysopids in olive trees that has motivated their study, but also in part, by the extended use of McPhail traps for monitoring *B. oleae*, which, baited with ammonium phosphate, turned out to be an excellent mean for capturing adult green lacewings in considerable numbers. The use of this sampling method and others has served to characterize the species assemblages in the top olive producing countries of the Mediterranean basin i.e. Spain, Greece, France, Italy and Lebanon. Szentkirályi (2001b) carried out a complete revision of the results obtained in different countries finding that up to 32 different species of chrysopids may be found in Mediterranean olive orchards (Table 1.1); 13 in the Iberian peninsula, 16 in France, 12 in Italy, and 18 in Greece. From these results he extrapolated an expected species assemblage for a Mediterranean olive orchard from 7 to 10 different species.

The species of the *carnea*-complex were, as expected, present in all the surveys along with the species *D. flavifrons*. Other species highly extended and abundant across the Mediterranean olive orchards were *D. prasina* and *Dichochrysa zelleri* (Schneider). *Dichochrysa genei* (Rambur) was also present in almost all the orchards surveyed but registered a reduced abundance within the species assemblage. It is especially noteworthy *Rexa lordina* Navás, a species that has just been detected in olive orchards from the Iberian Peninsula and southern France (Campos and Ramos, 1983; Monserrat, 2008; Monserrat and Marin, 1994) but probably present also in Moroccan olive plantations, and for which a very high specificity towards the olive tree ecosystem has been observed. Indeed, the majority of the individuals cited of this species have been collected from olive orchards and most of the rest associated to Oleacea plants (Monserrat, 2008).

Table 1.1 Species composition and dominance distribution within the local chrysopid assemblages found in olives groves in the Mediterranean parts of Europe and Near East (Szentkirályi, 2001b).

	1	2	3	4	5	6	7a	7b	8	9a	9b	10	11	12	13	FR
Chrysoperla carnea s. l. (Stephens)	47.9	61.2	6.7-25.3	39.3	23.8-	48.0	42.7	42.0	1.5	13.1	31.0	17.2	+			13
Chrysoperla mediterranea (Hölzel)			0-1.6													1
Chrysoperla mutata (McLachan)							+									1
Chrysopa formosa Brauer	0.8	0.1	0-0.1		0-6.4								+			5
Chrysopa pallens (Rambur)		+	0-0.4		0.7.1		+	+		+		0.8	+			8
Chrysopa nigricostata Brauer													+			1
Chysopa viridana Schneider	1.1	0.1	0-0.9							+		+	+			6
Chrysopa dubitans McLachlan											1.8		+			2
Dichochrysa prasina (Burmeister)	18.3	3.4	42.3-	1.7	14.9-	7.6	+						+			8
Dichochrysa flavifrons (Brauer)	15.6	24.2	11.6-	22.2	0-9.5	37.2	5.3	2.4	65.8	50.2	37.1	13.7-	+			13
Dichochrysa ariadne (Hölzel)												+	+			2
Dichochrysa picteti (McLachlan)	4.6	2.2	0.3-9.5	12.8												4
Dichochrysa zelleri (Schneider)			2.2-7.4	0.9			32.3	17.6	20.2	32.9	15.1	28.9	+		+	10
Dichochrysa clathrata (Scheneider)				9.4	0-6.4		9.7	13.6	1.6		+	+	+			8
Dichochrysa granadensis (Pictet)	0.8															1
Dichochrysa subcubitalis (Navás)	1.5	0.1														2
Dichochrysa genei (Rambur)	3.4	3.4					1.7	1.2	+	+	1.1	+	+		+	10
Dichochrysa nachoi Monserrat	2.3															1
Dichochrysa baetica Hölzel			0-0.9													1
Dichochrysa iberica (Navás)			0-0.7													1
Dichochrysa venosa (Rambur)		1.6												+		2
Nineta flava (Scopoli)			0-0.4													1
Cunctochrysa alboineata (Killington)													+			1
Cunctochrysa baetica (Hölzel)	2.7	1.5				7.0										3
Italochrysa italica (Rossi)			+	0.9	0-17.8				+				+		+	6
Italochrysa vartianorum Hölzel															+	1
Notochrysa capitata (Fabricius)			0-1.1	9.4												2
Brynckochrysa michaelseni (Esben-							+	+		+	4.1	+	+			6
Brynckochrysa nachoi (Monserrat)			0-1.3													1
Rexa lordina Navás	1.1	2.2	0-0.1													3
Rexa raddai (Hölzel)												+	+			2
Suarius nanus (McLachlan)							6.3	22.0	10.4	2.2	9.6	+	+			7

Note: The dominance values (%) were calculated from the number of collected individuals published in the reviewed paper. **Abbreviations:** +, presence of species, FR, frequency of species occurrence in literature surveyed. **Sources:** 1- Spain (Monserrat and Marin, 1994). 2- Spain (Campos and Ramos, 1983). 3- Southern France (Alrouechdi, 1984; Alrouechdi et al., 1980a; 1980b). 4- Italy (Liber and Niccoli, 1988). 5- Southern Italy (Pantaleoni and Curto, 1990). 6- Italy, Sardinia (Pantaleoni et al., 1993). 7a and b- Greece, Aguistri (Canard and Laudého, 1977). 8- Greece, Akrefnion (Canard and Laudého, 1980). 9a and b- Crete (Neuenschwander et al., 1981; Neuenschwander and Michelakis, 1980). 10- Western Crete (Canard et al., 1979). 11- Greece (Santas, 1984). 12- Lebanon (Heim, 1985). 13- Turkey (Segonca, 1981)

Little is known on the species assemblages in olive orchards outside the Mediterranean region. However, green lacewings are known to be present in Californian olive orchards (Daane et al., 2011) and it is highly presumable that several North American *Chrysoperla* spp. are part of the fauna of Californian olive trees. In South American there is a sole report on the green lacewing assemblages corresponding to the Argentinean zone of La Rioja. Six species were found; *Chrysoperla asoralis* (Banks) (59.6%), *Chrysoperla argentina* González Olazo and Reguilón (24.6%), *Chrysoperla externa* (Hagen) (3.5%), *Ungla argentina* (Navás) (5.3%), *Ungla binaria* (Navás) (5.3%) and *Ceraeochrysa claveri* Navás (1.8%) (Olazo et al., 2011).

Far less studied is the diversity of larvae present on the tree canopy. In fact, almost all the studies listed in Table 1.1, 3 out of 13, have used McPhail trapping to sample chrysopids neglecting the information on larval ecology which is the most relevant for the biological control role played by this group against olive pests. Neuenschwander and Michelakis (1980) observed that only three out of eight species collected from Cretan olive trees were actually reproducing on them and corresponded to the main species, *C. carnea s.l.*, *D. flavifrons* and *D. zelleri*. The species of the *carnea*-group were by far the most numerous doubling in abundance the two other species together. In southern French olive orchards *C. carnea s.l.*, *D. flavifrons*, *D. prasina* and *Chrysopa pallens* (Rambur) eggs were collected from olive trees, but as in Crete, *C. carnea s.l.*, dominated with 89.7% of the total oviposition (Alrouechdi et al., 1980b). More recent research has shown similar results in Sardinia where *C. carnea s.l.* larvae dominated accounting for 88.4% of the individuals collected through beating. *Cunctochysa* spp. larvae were found on the tree in addition to *C. carnea s.l.* and *Dichochrysa* spp. (Pantaleoni et al., 2001). From the few studies that have sampled immature stages it has been deduced that only species of the carnea-group seem to show a strong preference towards olive canopies for oviposition and their larvae are the main occupants of this stratum in olive orchards (Pantaleoni et al., 2001).

As mentioned above, lacewing larvae in olive crops have been extensively related to the natural pest suppression of several pests with different potential to produce economic harm. Homopteran pest insects have

been linked to chrysopids presence and predation in different Mediterranean locations (Alrouechdi et al., 1981; Argyriou, 1967; Liber and Niccoli, 1988). Alrouechdi (1981) found higher levels of black scale infestation an higher amounts of honeydew production linked to an increase in chrysopids adults and higher chrysopid oviposition rates. *E. olivina* has been also connected to chrysopids presence throughout literature (Alrouechdi et al., 1980a; 1980b; Alrouechdi et al., 1981). Thus, it is not unusual to find chrysopid larvae, within and near accumulations of the cottony secretion produced by nymphs of this minor pest, exhibiting a greenish coloration indicative of the possible consumption by the predator of immature psyllids (Porcel, personal observation). Another homopteran, the scale *Aspidiotus nerii* (Bouché), has also been regarded as a possible prey for lacewings in Cretan olive orchards (Canard et al., 1979; Neuenschwander and Michelakis, 1980). It has been shown that the increase in adults according to higher homopteran infestations is mediated by honeydew secretions which are attractive and can be used as supplementary food by chrysopids. Indeed, the attraction towards *S. oleae* honeydew has been suggested by Alrouechdi et al. (1980a) and Liber and Niccoli (1988) and artificial honeydew has proved to be attractive to olive-dwelling *Chrysoperla* spp. adults (McEwen et al., 1994). Despite this attraction, *S. oleae* immature stages may not be a completely suitable prey as it is believed that chrysopids are unable to complete their normal development by feeding on this prey alone (Morris et al., 1999a). This is not the case of *E. olivina*, a prey able to fulfill the nutritional requirements of *C. carnea s.l.* conferring both high survival and normal adult reproductive capacity (Porcel et al., unpublished).

Chrysopids have also been extensively related to *P. oleae* as one of the main natural regulators of this major pest (Alrouechdi et al., 1981; Campos and Ramos, 1983; Neuenschwander and Michelakis, 1980; Ramos et al., 1978). Alrouechdi (1981) tested at laboratory level *C. carnea s.l.* predation upon *P. oleae* eggs observing that all three instars were able to consume eggs showing a positive functional response. *C. carnea s.l.* is also able to handle and feed on forth instar larvae of *P. oleae*. Predatory first instars find difficulties in doing so, but bigger *C. carnea s.l.* larvae (second and third instars) feed on *P. oleae* showing excellent survival, development and

reproduction rates (Porcel et al., 2010). At field level, extensive field work in different Mediterranean zones has confirmed a correspondence between chrysopid adults and larvae peaks, and the presence of antophagous larvae and eggs of the carpophagous generation, in the months of June and July (Alrouechdi et al., 1981; Campos, 1989; Neuenschwander and Michelakis, 1980; Pantaleoni et al., 1993). In addition, the prey predator relationship has been confirmed through serological tests carried out on *C. carnea s.l.* individuals collected at the time the prey was present (Morris et al., 1999b). Studies on *P. oleae* eggs predation have detected eggs consumption in all of the three generations of the moth (Ramos and Ramos, 1990), however, the most relevant predatory activity of economic importance is carried out on the eggs carpophagous generation (Campos, 2001). In olive orchards from Southern France and Italy the predation rates of carpophagous eggs ranged from 7% to 45% depending on sites and years and incrementing as a function of infestation. However, this predation is effective in pest control only when all the eggs from the same olive fruit are destroyed as a sole larva is able to cause the fruit to fall. Thus, the measure of protected fruits ranged in these sites from 6% to 25% and again depended on the pest infestation (Alrouechdi et al., 1981; Liber and Niccoli, 1988). These moderate predations and olive protection values highly contrast with the observations made in Andalusian olive orchards. *P. oleae* carpophagous eggs predation rates in studies carried out during twenty consecutive years ranged between 19% and 97% with a mean values for the whole period of 71%. An average of a 57% of the olive fruits were protected from the negative effects of the pest (Ramos and Ramos, 1990).

It is noteworthy to mention that all these observations of *P. oleae* eggs predation are based on counts of the remains of the eggs sucked from the inside encountered on olive fruits, flowers and leaves. Researchers have for long associated this predatory activity to chrysopids, and mainly to *C. carnea s.l.* due to its supremacy on the olive trees canopy. However, it has been observed that such exclusive association neglects the possible activity of other sucking predators, mainly heteropterans, which are known to occur in olive orchards as well as chrysopids (Morris et al., 1999b; Pantaleoni et al., 2001). Furthermore, Morris et al. (1999b) suggested that a number of chewing predators might also consume *P. oleae* eggs leaving no evidence of their

predatory activity behind. Due to the small size of the eggs, piercing-sucking type predators are most likely their major consumers. *C. carnea s.l.* has been proved to be involved in *P. oleae* eggs predation, however, almost nothing is known on the predatory role of heteropteran species (e.g *Anthocoris* spp.*)* and other chrysopids such as *Dichochrysa* spp. which have been associated to *S. oleae* (Szentkirályi, 2001b). The possibility of a complex of *P. oleae* eggs consumer species is highly plausible and should be the aim of future reseach.

It may be concluded that the action of chrysopids in the olive agricultural ecosystem is substantial in terms of pest control and damage reduction and therefore, for the sake of crop protection, their natural populations should be conserved and promoted in order to increase their positive influence (Campos, 2001).

1.6. References

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2. Objectives

The objectives of the present doctoral thesis are:

Objective 1: To develop a laboratory methodology set up using the automated video tracking system EthoVision XT, with the objective of characterizing *C. carnea s.l.* larval behavior using precisely calculated movement parameters.

Objective 2: The evaluation of both the biological and behavioral effects of the use of kaolin particle films as a pest control method in olive orchards on the predator *C. carnea s.l.*

Objective 3: To establish the effect of insecticide usage and the herbicidal removal of weed cover on chrysopid abundance and diversity and in olive orchards and to assess the response of chrysopids to different management systems in relation to these agricultural practices.

Objective 4: To determine the effect of managed resident vegetation cover in olive orchards on the abundance and diversity of green lacewings and their availability for biological control of *Prays oleae* (Bernard).

Objective 5: To mark internally *C. carnea s.l.* adults by using oil soluble dyes and to assess any possible adverse effects on biological and behavioral parameters of marked individuals.

3. General Material and Methods

3.1 Study sites

The field case studies, conducted as part of this thesis, were carried out in different olive orchards from the province of Granada located in southwestern Spain within the Regional Authority of Andalusia. These olive orchards belonged to the municipalities of Deifontes, Colomera and Moclín (Fig 3.1) situated in regions with an old olive-growing tradition, the "Montes Orientales" and "La Vega" regions.

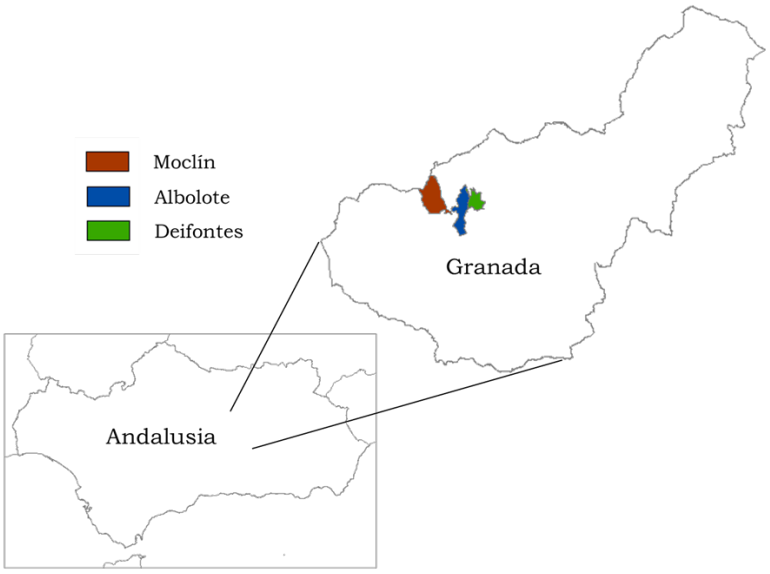

Figure 3.1 Location of the sampled olive orchards in the region of Andalusia.

The study zone has a continentalized Mediterranean climate characterized by marked temperature fluctuations with relatively cold winter and long and warm summers. During the winter month, snow events are not uncommon and the average temperatures are usually between 6 and 7º C as a consequence of the altitude. In contrast, summer temperatures average 20º C and the warmer month, July, reaches an average value of 34.4º C. Precipitation is in general low with a mean value for the province of Granada of 357 mm and distributed unevenly throughout the year. Rain events are concentrated from October to May whilst summers are extremely dry especially in the month of July and August when precipitation are often below 5 mm. The climate is also characterized by a strong interannual rainfall variability that determines intermittent draught periods. The landscape

configuration consists mostly of hills cultivated with olive trees and some annual crops with scattered highlands of uncultivated land occupied by natural or reforested woodlands. The lowlands of "La Vega" are highly fertile agricultural fields where a variety of vegetable and forest crops have been traditionally cultivated.

Olive cropping is known to be traditionally conducted in the zone since the middle ages, and therefore, a variety of cultivars, old and new, such as Picual, Picudo Hojiblanca, Escarabajuelo, Manzanillo and Gordal de Granada coexists in the agricultural landscape. According to Guzmán-Álvarez (1999), the olive orchards of this region may be mostly classified under conventional management due to their high level of agricultural intensification.

Ten total olive orchards have been used for the research conducted in the present thesis. Nine different olive orchards were used to carry out the study described in chapter 6 (Fig. 3.2). Three orchards were sampled during 1999 and 2000, and the remaining 6 plus the other 3 sampled in 1999-2000 (9 orchards in total) were investigated in the year 2003. A different orchard was used in the experiment carried out in 2009 and 2010 described in Chapter 7. The general characteristics of each individual orchard are detailed below according to farmer's information and weedy plants identification given in Cárdenas (2009). The olive orchards coded OR1, IT1 and CV1 were sampled in the years 1999 and 2000 from March to October covering the full period of chrysopid activity in this crop (Chapter 6). The orchards OR1-3, IT1-3 and CV1-3 were sampled in mid-May and mid-June 2003 (Chapter 6). Finally, IT4 was used to assess the impact of vegetation cover on chrysopids from May to September 2009 and 2010. All the orchards are listed below classified attending to their management system.

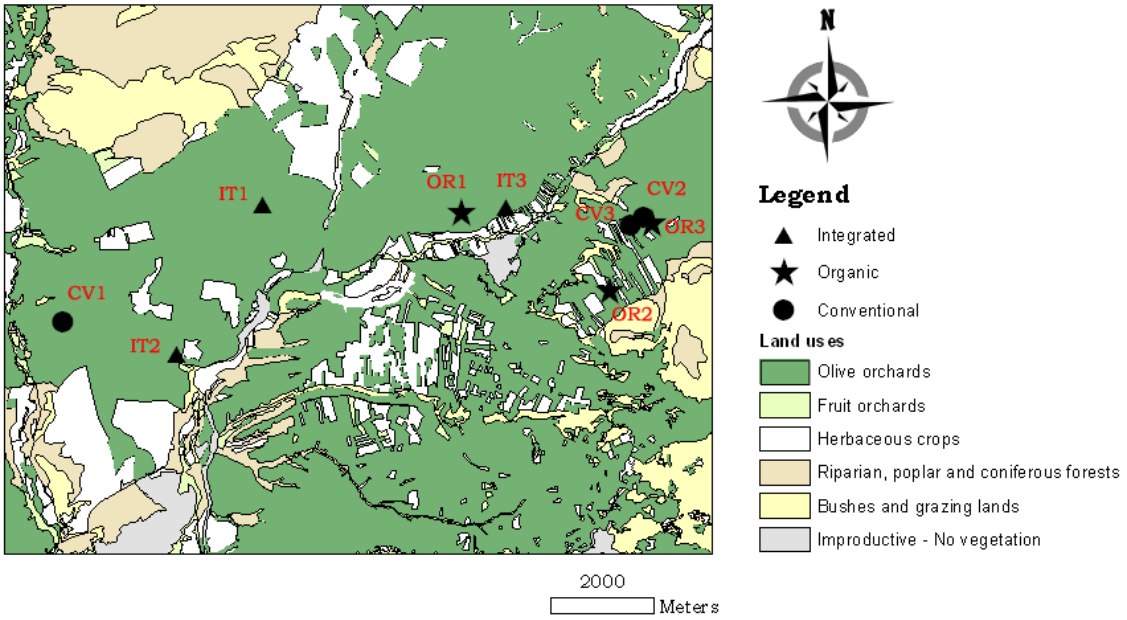

Figure 3.2. Location of the orchard sampled in 1999, 2000 and 2003 (Chapter 6) and surrounding landscape composition.

Organic orchard 1 (OR1): This orchard is part of a larger area known as "Loma del Galgo" (Fig. 3.3) belonging to the municipality of Deifontes and situated at 446692.69E 4132239.70N and 821 masl. The orchard is quite steep with a mean slope of 27.6% and has a total extension of 2.24 ha. Olive trees were 50 years old of the "Picual" cultivar and irrigated during rainfall scarcity. The orchard had resident vegetation cover comprised mainly by the species *Gallium* sp., *Lentodum* sp., *Geranium* sp., *Medicago arabica*, *Coronilla scorpioides*, *Calendula* sp., *Erodium* sp., *Hordeum* sp. and *Raphanus* sp. Organic fertilizers were applied at a rate of 2 L per tree and shallow plowing was carried out twice a year.

Organic orchard 2 (OR2): This orchard is situated in an extensive olive-growing zone called "Atalaya" located also in Deifontes at 449549.72E 4130741.18N and at an altitude of 1035 masl. The orchard was moderately steep (9.3%) and covered an area of 1.52 ha. The olives were relatively young with around 12 years old of the "Picual" variety and under irrigation. Soil management included the maintenance of a vetch (*Vicia sativa* L.) cover crop during spring. Fertilization was carried out with natural products.

Organic orchard 3 (OR3): The orchard is part of the olive-growing zone known as "La Rambla" also in the municipality of Deifontes. Its situation is 450388.71E 4132048.45N and has a moderate slope of 6.8% and a surface of 0.96 ha. Olive trees are of the "Picual" cultivar and irrigated during the summer. Natural fertilizers were applied to the soil that was also plowed twice a year. The orchard had vegetation cover dominated by the weedy species *Medicago rigidula*, *Erodium* sp., *Hordeum* sp., *Erysimum* sp., *Capsella* sp., *Calendula* sp. Additionally, there was a hedgerow adjacent to the orchard.

Integrated orchard 1 (IT1): The orchard is called "Arenales de San Pedro" (Fig. 3.4), situated in a zone known as "Laguna Vieja" located in the municipality of Albolote. Its coordinates are 442864.06E 4132384.84N and it is situated at 751 masl. The orchard is rather extensive (57.0 ha) and flat (4.2%). The olives were big in size, of the Picual cultivar, and about 80 years old. The orchard was flood irrigated twice a year and had neither managed vegetation cover nor adjacent hedgerows. Herbicide and insecticide were used, mainly dimethoate and simazine and deep plowing was carried out.

Figure 3.3. Orchard located in "Loma del Galgo", Deifontes (OR1).

Figure 3.4. “Arenales de San Pedro” (IT1).

Integrated orchard 2 (IT2): This orchard is located in the zone “Cortijo Cajil” in Albolote at 441190.07E 4129513N. Situated at 745 masl, has a moderate slope of 7.6% and a considerable extension of 256.6 ha. The orchard has trees of different ages; however, the trees sampled were about 12 years old and belonged to the variety Picual. Olive trees were drip irrigated. The presence of weedy plants was usual in between tree rows i.e. *Medicago arabica*, *Anagallis* sp., *Hordeum* sp., *Lentodum* sp, *Plantago* sp. *Anagallis* sp., *Scorpiorus* sp., *Euphorbia laxa*, *Geranium* sp., *Erodium* sp., *Trifolium* sp. and *Calendula* sp. Herbicides were used for weed control but no insecticides were applied during the sampling years. Neither plowing nor chemical fertilization were carried out during the sampling season.

Integrated orchard 3 (IT3): The sampled orchard belonged to the zone known as “Loma del Perro” situated in Deifontes. Situated close to the railway station at 751 masl and with UTM coordinates 447575.81E 4132355.94N, covered a total surface of 0.612 ha. The area was hilly and the slope of the orchard steep (26.8%). The trees were old and planted following a quincunx

pattern. The orchard was not irrigated and neither vegetation cover nor hedgerows were present within it. Herbicides were applied and plowing carried out as part for soil management. Chemical fertilizers were commonly used and pesticides when required.

Integrated orchard 4 (IT4): This orchard is known as "Finca de Enmedio" and it is situated in the municipality of Moclín but closer to the villages of Pinos Puente, Tiena and Olivares. The situation of the central point of the orchard in UTM coordinates is 431223.94E 4128326.30N and it is roughly between 650 and 700 masl. The orchard is very large, spreading over 235 ha, and the olive trees belong exclusively to the cultivar Picual and are around 90 years old, planted at a density of 110 to 130 trees per hectare. No soil plowing was carried out and soil management included the maintenance of vegetation cover situated outside the canopy, growing in at least 1.5 m wide strips that senesced naturally in June (Fig. 3.5). Herbicides were applied under tree canopies and the olive trees were chemically fertilized. No insecticide applications were carried out during the sampling years. Further information on soil management and vegetation cover detailed composition are provided in Chapter 7.

Conventional 1 (CV1): The orchard, known as "Cañada de la Laguna" (Fig. 3.6) is situated in Albolote at 438986.15E 4130127.82N and 715 masl and has an extension of 10.9 ha and a mean slope of 4.1%. The olive trees belong to the variety "Picual" and were approximately 60 years old producing a mean yield of 100 kg of olive fruits per tree. The orchard was drip irrigated fortnightly and chemical fertilization was carried out after harvesting. Dimethoate and α-cypermethrin insecticides were systematically applied as well as the herbicide simazine sprayed twice a year. The soil was deep plowed at least once a year.

Conventional 2 (CV2): This orchard belongs to the olive-growing zone of "La Rambla" situated in Deifontes, at the base of Sierra Arana at 1013 masl and at the coordinates 450238.18E 4132114.74. The orcahrd's slope was

moderate with an average value of 7.9% and it covered an extension of 2.0 ha. The olive tree variety was Picual displayed closer than usual, 8×8 m apart, and were very young, around 9 years old. Plowing was not usual in the orchard, however, herbicidal treatments were conducted against weeds preventing the establishment of vegetation cover and no hedgerows were present close to the orchard. Fertilization was carried out with chemical fertilizers and pest control using pesticide applications.

Conventional 2 (CV3): The orchard, also situated in "La Rambla" olive-growing zone in Deifontes, is located at 449981.29E 4131990.40N at an altitude of 1028 masl. The orchard's total surface is 2.0 ha and it slopes steeply (24.7%). The olive cultivar present is Picual. The trees were drip irrigation and lacked both vegetation cover (controlled by herbicides) and adjacent hedgerows. No plowing was carried out and pesticides were applied systematically throughout the year.

Figure 3.5. "Finca de Enmedio" orchard (IT4), Moclin. Aspect of vegetation covers senescing in late June.

Figure 3.6. "Cañada de la Laguna" (CV1), Albolote.

3.2. Field sampling techniques and experimental designs

In the different experiments conducted as part of this thesis, green lacewings have been sampled with a variety of field techniques, either for the capture of adults alone, or for the collection of both adults and larvae. Additionally, in chapter 7 the presence of *Prays oleae* (Bernard) eggs on olive fruits was monitored in order to determine the possible temporal coincidence with their predator and any variability among sampling zones. Below, the different sampling techniques employed are described in detail as well as the field experimental design of the plots (or blocks) corresponding to each technique. The number of plot (or block) replications for each year and sampling methods are given in Chapters 5, 6 and 7.

Tree-beating method: This technique was used to collect both adults and larvae from olive canopies during the years 1999, 2000 and 2003 for the study described in Chapter 6. This beating methodology was firstly applied to sample

olive trees in the fashion described below by Ruano et al. (2004). A group of branches, always of similar size and situated at about 1.5–1.75 m high, were introduced inside a large plastic bag and strongly shaken five consecutive times. The whole process was repeated four times per tree (one per cardinal direction). In order to prevent any interaction among the collected arthropods inside the bag, which could have resulted in a loss or damage of the entomological material, a quarter of a DDVP® (Dichlorvos) insecticidal tablet was introduced inside in order to inactivate the arthropods.

The experimental design followed the graphicall representation provided in Figure 3.7. Five trees situated in the same row were sampled leaving an unsampled tree between two sampled trees, and therefore, an approximate separation of 20 m between sampled trees. Each group of five trees was regarded as a sampling unit (or block) and a minimum separation of 500 m was established between units in order to ensure the spatial independence of samples.

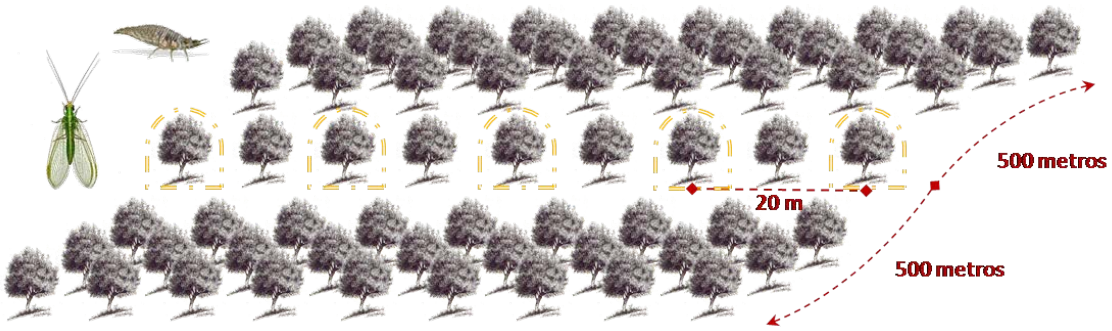

Fig. 3.7. Experimental design of the sampling units in 1999, 2000 and 2003 sampled through the beating method.

McPhail trapping: This method was used to sample adult populations during the years 2009 and 2010 (Chapter 7). McPhail traps have been traditionally used to establish chrysopid assemblages and populations in olive orchard from different countries (Alrouechdi et al., 1980; Canard et al., 1979; Corrales and Campos, 2004). The system provides the advantage, compared with other sampling techniques, of capturing an important number of adult

individuals per trap. McPhail traps were baited with an aqueous solution of 5% diammonium phosphate as an attractant plus 2% Borax® for insects conservation. The traps were hung between 1.5 and 2.0 m high in the inner side of the tree always facing north and were deployed in groups of five forming a quincunx pattern (Fig. 3.8). Hence, traps from the same group were separated by a distance of 14 m and groups of five traps, denominated plots, were separated among them by a minimum distance of 150 m. In the field the content of each trap was filtered with a nylon mesh obtaining on its surface the arthropods captured. Each nylon mesh was kept inside a plastic recipient to be taken to the laboratory.

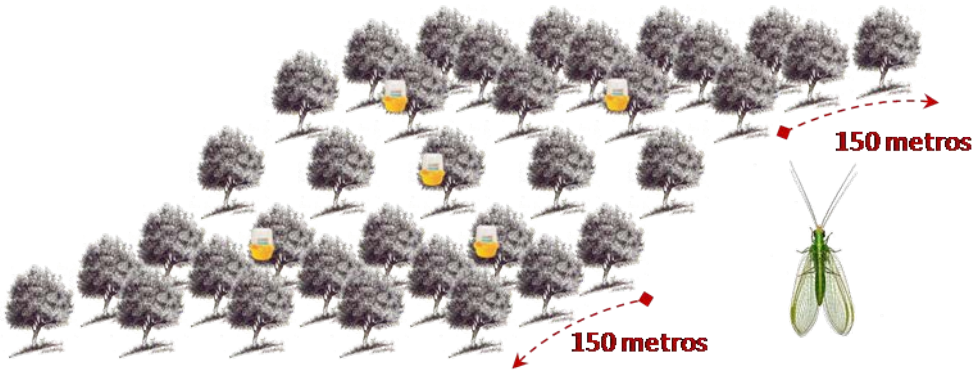

Fig. 3.8. Experimental design of the McPhail trap plots established in IT4 in the years 2009 and 2010.

Suction sampling: Olive canopies were suctioned, initially, in order to sample chrysopid larvae developing on the tree in the years 2009 and 2010 (Chapter 7). However, the method is also effective at collecting adults resting during the daylight hours on the tree's foliage and therefore was also used in the field case study described in Chapter 5. Suction sampling was carried out by using a commercial insect aspirator (Modified CDC Backpack Aspirator Model 1412, John W. Hock Co., Gainsville, FL, USA). Inner and outer branches of the olive tree were suctioned up to a height of 2 m for a period of 2 min by moving around the tree in order to cover all possible angles. The suction samples were collected inside customized socks that intercepted the insects dragged by flow produced by the aspirator. Each suction sampling plot consisted in a square plot of 16 contiguous trees (4×4) (Figure 3.9) and was regarded as an experimental unit. Suction sampling plots were separated by a

minimum distance of 100 m and located in different zones that the McPhail trapping plots. The samples were immediately introduced inside a cold box full of ice to prevent, as in the case of beating samples, the interaction among the captured arthropods before arriving to the laboratory.

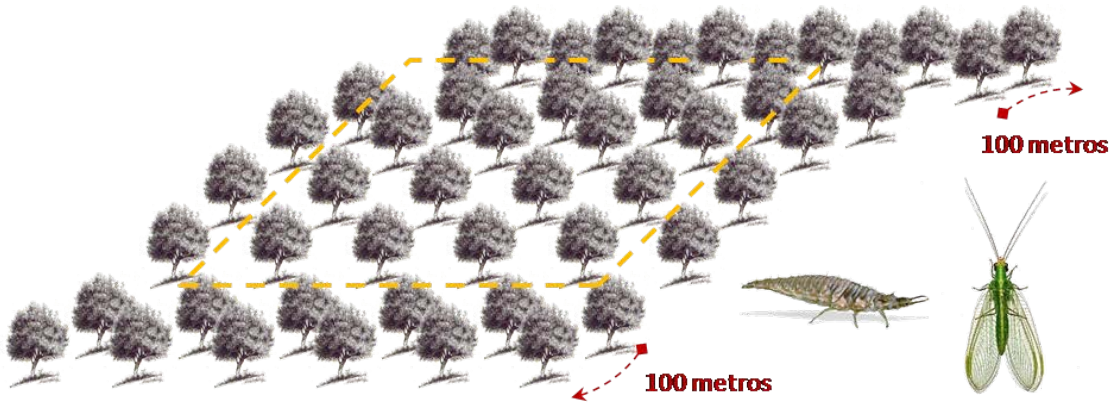

Figure 3.9. Experimental design of the plots of suction sampling carried out in IT4 in the years 2009 and 2010.

P. oleae egg monitoring: In the years 2009 and 2010, a survey was carried out on the eggs of the carpophagous generation of *P. olaea* (Chapter 7). Ten medium size twigs were selected from each olive tree. Two olive fruits were collected from each one of the selected twigs adding up 20 fruits per tree. The eggs collected from each tree were regarded as an experimental unit. *P. oleae* egg monitoring plots consisted on five trees displayed in a quincunx pattern (Fig. 3.10) and a total of 100 fruits were collected from each plot. Plots were separated by a minimum distance of 20 m and some of them coincided spatially with the location of McPhail trapping plots.

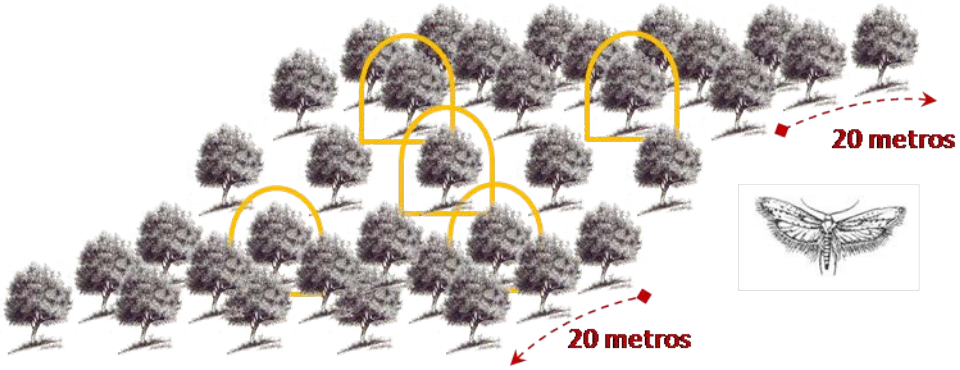

Figure 3.10. Experimental design of *P. oleae* eggs sampling plots in IT4 in the years 2009 and 2010.

3.3. Laboratory processing of field samples

Beating samples: On arrival from the field, the samples contained in bags were deposited inside big size horizontal refrigerators at –20º C for their optimal consevation. Sequentially the plastic bags were opened and cleaned from vegetal and other inert remains. The arthrpods were carefully collected using entomological forceps and fine camel paintbrushes using the stereoscope (Stemi SV8; Zeiss, Oberkochen, Germany) when required. Chrysopid larvae and adults were sorted from the rest of arthropods and kept inside Eppendoff tubes containing 70º ethanol.

McPhail samples: The plastic recipient containing the filtering mesh with the insect samples was also kept in horizontal refrigerators at –20º C. Green lacewing adults were separated from other insects (mainly the olive fruit fly, *P. oleae* and other moths, and wasps) and introduced in Petri dishes that were placed in the refrigerator for later identification.

Suction samples: On arrival, the samples were conseved in refrigerators at –20º C. The sample processing consisted on emptying the sample-containing socks on small trays and, under sterescope, carefully separating larvae and adults Chrysopidae from other arthropods and debris and placing them inside Eppendoff tubes for later identification.

P. oleae egg count: The eggs deposited on the olive fruits by anthopagous *P. oleae* adults were counted shortly after fruits collection in order to prevent their disruption. For the egg count, the remains of the calyx near the peduncle of the olive fruit was thoroughly revised under stereoscope as well as other adjacent parts of the olive fruit where *P. oleae* may also occasionally oviposit.

Once individualized the chrysopids from the rest of inert and entomological material collected with the samples, larvae and adults were identified taxonomically under stereoscope. Adults were identified to species level using the training provided by the chrysopid taxonomy specialist Dr Monserrat in the specialization course titled "Taxonomía de Crisópidos Ibéricos". All the adults of the *carnea*-complex of sibling species, except *Chrysoperla mediterranea* (Hölzel) were placed under the category of *C. carnea s.l.* (also referred in some parts of this thesis as *C. carnea*). Chrysopid larvae were identified to genus level using the keys provided by Díaz-Aranda et al. (2001). All the problematic adult and larvae individuals and rare species identifications were later confirmed by Dr. Monserrat.

3.4. The EthoVision system and the parameters used

EthoVision® is a video tracking system design for the automation of behavioral experiments. Video tracking systems were introduced in the early 90s, offering more flexibility, spatial precision, and accuracy than the techniques employed before for behavioral experiments with insects (Noldus et al., 2002). The system is comprised by several devices, including a computer, and specific software. The basic functioning of the system is as follows (Noldus et al., 2001): A CCD video camera records the area (denominated arena) in which the individuals are. The analog video signal is digitized by a frame grabber and passed on to the computer's memory. The software installed in the computer is then used to analyze each frame recorded by the camera in order to distinguish the tracked individuals from the background. This identification can be carried out based either on their gray scale (brightness)

or their hue and saturation (color) values. In the experiments conducted for this thesis the selected option for identification was gray scaling. Once detected the individual, the software determines the position of its center (center of gravity) in each video frame and saves this information to a track file for each recording session. Based on these values, the software is able to carry out calculations analyzing the whole series of video frames and produces quantified measurements of the insect's behavior. In addition, the software offers the option to identify certain regions as being of interest and allows calculating the variables in these regions individually.

The detailed description of the parameters calculated for the behavioral experiments conducted in Chapters 4, 5 and 8 is given below according to Noldus Information Technology (2007). These parameters are expressed as mean values for the whole trial in the referred chapters.

Distance moved: The length of the vector connecting two sample points (i.e., the distance of the center of gravity of the tracked animal between one sample and the next). This is calculated using Phythagoras' theorem.

Velocity: Distance moved per time unit (i.e., speed).

Distance to point: The distance between the center of gravity of the tracked animal and a location inside or outside the arena defined by the user.

Distance to zone: The shortest distance between the center of gravity of the tracked animal and the border of a user defined zone.

Total time spent or duration in zone: Whether or not the animal is in a particular zone of interest determined by the user.

Angular velocity: Speed of change in direction of movement (i.e., amount of turning per unit of time, absolute or relative). The angular velocity of each sample is the turn angle for that sample, divided by the sample interval.

Meander: Change in direction of movement relative to the distance moved (i.e., amount of turning per unit distance, absolute or relative). The mean of each sample is the turn angle for that sample, divided the distance moved from the last sample.

Movement: Whether or not the tracked animal's velocity exceeds a user-defined level. The user sets thresholds for both "start velocity" and "stop velocity," and the parameter is calculated over a running average of a user-defined number of samples. The parameter has two states: "moving" and "not moving."

The experimental design for the adaption of the EthoVision system for behavioral studies of chysopid larvae is fully detailed in Chapter 4. The methodological description of the use of the EthoVision system for the calculation of the parameters of a classic flight mill for *C. carnea s.l.* flight performance assessment is given in the following section.

3.5. *C. carnea s.l.* flight mill experiment

This section describes in further detail the methodology employed for the flight performance experiment, included as part of Chapter 8, which had the objective of testing whether dye-marked *C. carnea s.l* adults had the same flying ability as unmarked individuals.

A sufficient number of recently laid eggs coming from the main culture were transferred to a cabinet at at 25±1°C, 50–60% RH, and a photoperiod of 16:8 (L:D) h that had three different trays. In this cabinet, two photoperiods with the same length were set up in different trays with a 5 h time lag between them. The trays were also programmed to simulate dawn and nightfall in 30 min period each. To avoid light contamination among the trays, the third tray of the cabinet, situated between the other two, was kept in dark. In these conditions the insects were developed to adulthood. The recently emerged adults were collected for the experiment during the 30 min nightfall simulation the same day on emergence, coinciding with the stimulus that triggers the beginning of the migratory flight activity as observed by Duelli (1980).

The flightmill consisted of a 90 mg styrene 15.5 cm rotating arm (Evergreen Scale Models, Kirkland, USA) adhered at halfway point to the centre of a 38 mm entomological pin acting as a central axle. At one end of the rotating arm, a 2 mg, 9 mm long copper wire was glued perpendicularly to sustain the lacewing. An opaque black coloured paper card (8 × 12 mm, 7.5 mg) was attached to the other end, with the double objective of acting as a counterweight for the insect and providing an excellent detection source for the EthoVision software. The card size was therefore selected in accordance to the mean weights of adult lacewings, making sure that it had an enough surface for a successful detection. The whole rotating assembly (pin, arm, wire and card) was placed with its axle suspended between two magnets. The lower magnet contacted the pointed end of the pin and the upper one kept the pin in a vertical position without contacting its top to minimize friction. The two magnets were situated opposite two each other, embodied in a translucent methacrylate box opened to one side to allow the manipulation of the removable rotating assembly, and restricting the air movement at the same time (Fig. 3.11).

The box containing the flight mill was situated underneath the video camera (Fig. 3.11). The experiments were carried out in a controlled room illuminated by a single fiber optic illuminator 3 m above the arena and fitted to the ceiling. The objective was to attain a night illumination, under 10 lux as described in Duelli (2001), without compromising the object detection. Finally, a measured light intensity in the arena of 2 lux was achieved.

Adult lacewings were tethered to the copper wire using an extra fast formulation wood (Ceys S.A, Barcelona, Spain) adhesive. The tip of the wire was carefully attached to the insect's pronutum making sure that no glue made contact with head, antennae and wings obstructing the insect's movement. The first attempts to attach the lacewings to the mill revealed that this operation could be performed without anaesthetizing the insects due to their tendency to freeze as they noticed the wire contacting their pronotum with a certain amount of pressure. A special care was taken to ensure that the lacewing body was situated in a horizontal position with respect to the wire

preventing as far as practicable any deviation in the flight angle. The lacewings were free to fly and rest while hanging from the wire's tip. Once attached the insect, the rotating assembly was ready to be situated in its position between the magnets to initiate the trial

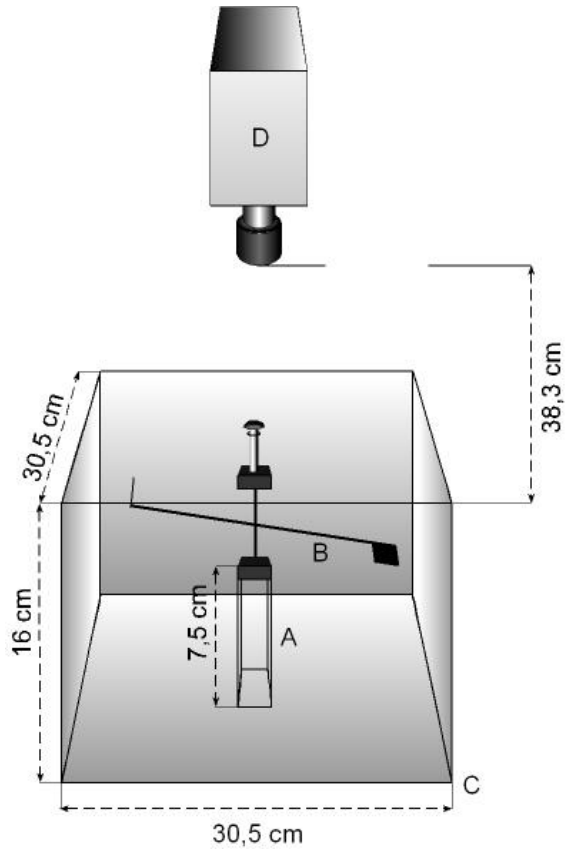

Figure 3.11. Flight mill video tracking experimental setup. (A) Magnets and its supporting structure, (B) rotating assembly, (C) plexiglass box, (D) video camera.

The circular movements of the mill were recorded and transmitted to the EthoVision system. For the experiment, the arena was design as two concentric circles, defining the area between them as the zone of interest and allowing the object detection only within this zone. An image captured with the camera from its overhead position was taken as reference. This area contained both ends of the rotation arm all along the circular trajectory of the mill. The central structure of the flight mill, comprising the two magnets and the rotation axle, remained within the smaller circle (9.5 cm diameter), outside the zone of interest preventing from object detection interferences. The larger circle (17.5 cm diameter) delimitated the outer boundary of the arena. As appointed before, the detection was focalized on the dark colored card

attached to the end of the rotating arm opposite to the lacewing's position. The parameters recorded for this alternative object, understandably, had an exact correspondence with those of the tested lacewing. The high contrast with the background produced by the card enhanced the detection, making easier to adjust the detection settings under the arranged light conditions. The use of this alternative object was adopted in view of the fact that a successful detection of the flying lacewing, at the appropriate camera distance, proved to be highly problematic. The subtraction object detection method and a dilation filter were selected giving the must optimal results in the experimental setup. The detection settings were accordingly adjusted and the rest of the settings were left as default.

The tested individuals were subjected to a 5 h flight mill recorded trial. Two adult lacewings were tested daily with individuals taken at random from the two photoperiods programmed cabinet. The trials were conducted at 9:00 and 14:00 in consecutive days until the completion of the experiment. At times, the lacewings attached to the mill initiated the flight whilst the structure was situated in place inside the box. In other occasions a soft air current was used as a flight eliciting-stimulus. A minimum of 2 min duration flight was considered as a sustained activity event, in opposition to a trivial flight (Blackmer et al., 2004). Trials with a less duration flight were discarded.

3.6. Statistical methods

This section describes the statistical methods applied in the different chapters of the thesis.

3.6.1. Data exploration and analysis assumptions

All the data sets were subjected to data exploration following Zuur et al.(2010) in order to avoid violations of the underlying assumptions of the statistical techniques employed. A correct data exploration is recommended as

it helps to reduce type I and type II errors and the chance of making wrong conclusions from the experiments outcome. Initially, outliers were eliminated from data sets. The outliers were detected using the boxplot, a graphical tool that is typically used for their identification, and were only removed in case there was an evident reason for their deviation from the rest of the values. No outliers were removed from field case studies datasets. Continuous data from laboratory and field experiments (e.g. larval weight and Shannon index) were checked for normality and homocedasticity (homogeneity of variances) as they are important assumption before the application of popular analysis as the analysis of variance (ANOVA and MANOVA) and Student *t* tests. Kolmogorov-Smirnov, Shapiro-Wilk tests (depending on the number of replications) and energy test of multivariate normality were used to check for the normality assumption and the Levene test and Box's test of equality of covariance matrices for the homocedasticity assumption. Some datasets were transformed to improve the fit to a normal distribution and homogenize their variances among treatments applying $\log_{10}(x + 0.5)$ and the arcsin (x) transformation for percentages. In the case of model construction using the normal distribution, homocedasticity and normality assumptions were checked by graphical representation of the model residuals versus fitted values and the residuals histogram respectively (Zuur et al., 2007).

In field studies datasets, the variables used for the construction of multiple regression models were check for collinearity (correlation between explanatory variables) using Pearson correlation coefficient. Nonetheless, no variables were found to be highly correlated enough to produce problems to the analyses.

An essential assumption of the majority of statistical techniques is the measurements independence (Hurlbert, 1984). This means that the information from any sample should not provide information on another sample after the effects of other variables have been accounted for (Zuur et al., 2010). In case this dependence is detected, the statistical models used to analyze the data need to account for it. The independence assumption has been taken into account for all the experimental design at laboratory level.

However, at field level two types of dependence situations are highly usual, spatial autocorrelation and temporal autocorrelation.

Samples taken from the same location may be closer in value than samples taken from locations farther apart. This is known as spatial autocorrelation and violates the independence assumption. The existence of spatial autocorrelations was not checked in datasets. On the contrary, it was directly assumed to exist in field experiments and corrected in the statistical analysis. This was carried out by including a variable accounting for it in the constructed models as an explanatory variable or a random effect. The option applied for each experiment is furthered detailed in the different chapters. Temporal autocorrelation refers to the correlation between sample values taken in a time series. It reflects the fact that the value obtained at a given time is not completely independent of its precedent value in the time series. The presence of temporal dependence was checked by plotting auto-correlation functions (ACF) for regularly spaced time series (Chapters 6 and 7). Whenever this dependence was detected, statistical analysis were conducted in order to account for them by using a residual correlation structure and including a smoothing function of time (Chapter 7).

3.6.2. Univariate methods and regression models

In experimental designs with a one or two explanatory categorical variable and a continuos response variable (i.e the laboratory experiments), parametric methods, such as ANOVA, were preferred as they produce more accurate and precise estimates. Data were transformed and parametric tests assumptions checked as described above for the analysis of all the laboratory experiments. If the datasets met the assumptions Student's *t*-test for comparing two treatments, and ANOVA for the comparison of more treatments were conducted. In case the assumptions were violated, non-parametric analysis had to be used. Hence, the Mann–Whitney *U* test was carried out for the comparison of two treatments and the Kruskal–Wallis one-way analysis of variance by ranks for comparing more than two independent treatments.

Several post-hoc tests were carried out to identify differences in means between treatments and the Bonferroni correction was applied whenever required.

For the analysis of continuos or discrete variables (count field data) as a function of several explanatory variables, generalized linear and additive models were applied. A regression model was also used for the study of adult survival in Chapter 8.

Linear regression is an approach to modeling the relationship between a response variable and one or more explanatory variables using linear functions. If there are more than one explanatory variable the analysis is called multiple regression. Thus, model regression estimates, standard errors, *t*-values and *p*-values are estimated for each explanatory variable from the dataset. In linear regression models, a mathematically identical test to an ANOVA is the single parameter *t*-test that contrasted with the *t*-distribution (with given number of degrees of freedom) produces a *p*-value. From this *p*-value it can be concluded whether the explanatory variable is significant or not. The mathematical sign (positive or nagative) of the regression estimate indicates whether the relationship of the explanatory variable with the response variable is negative or positive.

A generalized linear model (GLM) is a flexible generalization of ordinary linear regression that allows for response variables that have other than a Gaussian or normal distribution. This is achieved by allowing the linear model to be related to the response variable via a link function. The Poisson and negative binomial distributions (and not the Gaussian distribution) are the correct options for modeling count data (Zuur et al., 2009) which is the type of data mostly obtained in the field experiments of this thesis. The link used for Poisson regression is typically the logarithm. In most of the analysis of field experiments, extensions of the GLM analysis were applied in order to include a variety of effects. Generalized linear mixed models (GLMMs) extend the basic GLMs by including random effects in addition to the fixed explanatory

variables. The inclusion of random effects was used in the analyses to account and correct for spatial and temporal autocorrelation (Chapters 5, 6 and 7). Generalized additive models (GAMs) also extend GLMs by including another feature; these models allow for non-linear relationships between the response variable and all or part of the explanatory variables. This capability was used to model seasonal variation of chrysopids in Chapter 6, and generalized additive mixed models (GAMMs), that allow both non-linear relationships and random effects, were used for the same purpose in Chapter 7.

Finally, a Cox proportional hazards model was used in Chapter 8. This model is similar to those described above and relates the time that passes before some event occurs (*C. carnea s.l.* adult death in this case) to one or more explanatory variables (dye concentration and sex).

3.6.3. Multivariate methods

Multivariate methods have been employed for the analysis of datasets with more than one response variable. Three multivariate methods have been used throughout this thesis: Principal component analysis (PCA), multivariate analysis of variance (MANOVA) and a particular partial redundancy analysis known as principal response curves (PRC).

PCA is an unconstrained ordination method useful for identifying patterns in multivariate data otherwise difficult to observe. The graphical representation of this analysis (the PCA biplot) expresses the data in such a way as to highlight the similarities and differences among multivariate samples (dots) and their relationship with the measured variables (arrows). A mathematical transformation procedure generates from the original data a new coordinate system such that the greatest variance is contained in the several first axes. The other main advantage of PCA is that it compresses multivariate data by reducing the number of dimensions with, in many cases, an insignificant loss of information. Thus, in Chapter 4 this method was

applied for the graphical representation of the differences among treatments of a set of behavioral parameters measured by the EthoVision system. In Chapter 6 it was used with the same purpose, representing the differences among the chrysopid species assemblages among different orchards. In the species data obtained in the year 2003, the reduced first two dimensions of the analysis were used as synthetic variables of the whole species multivariate dataset and subjected to a MANOVA analysis. This analysis is similar to ANOVA but applied when there are two or more response variables. It differs from ANOVA in that it uses the variance-covariance between variables in order to test the statistical significance of the mean differences.

Redundancy analysis is also an ordination method but, unlike PCA, it is constrained, which means that explicitly relates two matrices: one response variables matrix and one explanatory variables matrix. The analysis is performed taking into account the set of explanatory variables. The ordination identifies which axes are best explained by a linear combination of the explanatory variables and therefore it may be considered the multivariate analog to the multiple regression models described above. A detailed description of the partial redundancy analysis PRC is given in Chapter 7.

3.7. Measurement of biodiversity

The biodiversity measures calculated in the present thesis (Chapter 6 and 7) have aimed to quanitify chrysopid diversity in different orchards and experimental zones and to establish its temporal evolution throghout the sampling period. The measures of biodiversity used are described below.

Species Richness: Usually notated as *S*, it is described as the number of different species part of a given set of biological individuals. In Chapter 6 it was calculated as the number of different chrysopid species represented in the complete data set in a particular olive orchard for the whole sampling period. Therefore, species richness is simply a count of species, and it does not take into account the total or realtive abundances.

Shannon index: This index, also known as the Shannon-Wiener and noted as H, is the most popular in ecological research which has the advantage of allowing comparison among different studies. The index is based on the information theory and, in ecological studies, measures the order observed within a particular site characterized by the number of individuals of each species. Thus, high values of the index represent a more diverse community and are related to high species richness and the even distribution of species abundances (values are rarely above 5). On the contrary, communities with only one species have a Shannon index value of zero. The index is calculated as follows:

$$H = -\sum_{i=1}^{s} p_i \ln p_i$$

where:

H = Shannon index

p_i = fraction of the entire population made up of species i

S = numbers of species encountered

$\sum$ = sum from species 1 to species S

Dominance: The dominance is defined as the fraction of the total collection of individuals that is represented by the most common species. This index accounts for the most abundant species without taking into account the contribution of the rest (Moreno, 2001). Dominance varies from 0, when all the species are equally represented, to the value of 1 when a sigle species is present in the dataset. This index is useful for assessing resource monopolization by a superior competitor in the particular case of the study of species communities with functionally redundant ecological roles such as chrysopids.

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4. Characterization of the locomotory activity of *Chrysoperla carnea* (Neuroptera: Chrysopidae) larvae using computerized video tracking in the presence and absence of a food source

Characterization of the locomotory activity of *Chrysoperla carnea* (Neuroptera: Chrysopidae) larvae using computerized video tracking in the presence and absence of a food source

4.1 Abstract

Chrysopid larvae are valuable predators in agricultural ecosystems whose main prey seeking mechanism involves important locomotory activity. Thus, movement capability is a key factor affecting their predatory performance. An experimental setup and methodology were design aiming to characterize *Chrysoperla carnea* larval behavior by means of the computerized video tracking movement analysis EthoVision XT. Larval movement parameters were recorded in the presence of different food sources and no food (control) in the whole arena and within a food influence zone. Data analysis clearly distinguish the control treatment from the rest. Additionally, some differences in behaviour were observed with honey as a food source compared to natural prey (lepidopteran eggs and aphids). The complete set of parameters extracted from the video analysis succeeded in characterising different *C. carnea* behavioural patterns.

Keywords: EthoVision, green lacewing, movement analysis, predator

4.2 Introduction

The chrysopids are a worldwide extended family occurring in most field crops and well known for their predatory activity against a wide range of soft-bodies pest insects of economic importance and their eggs (Duelli, 2001; Stelzl and Devetak, 1999). Among the different species, those belonging to the so-called *carnea* species complex commonly play a prevailing role in the biological control in crop environments. Due to its cosmopolitan distribution, its relevance as a foliage-dwelling predator and its easy rearing, it has been appointed as an important beneficial test species for regulatory requirements (Vogt et al., 2001). The larvae of this predator are quick and active searchers seeking their prey on crop plants with an eventual prey encounter happening at random, just influenced slightly and within short distances by honeydew and lepidopteran scales (Canard, 2001). Among the several strategies available to increase the probability of finding suitable prey in a lack of information situation, this behavior falls under the category of random search. Low prey density situations are quite usual in agricultural ecosystems where chrysopid predatory activity is effective. This is the case of the olive moth, *Prays oleae* (Bernard), that produces egg densities as low as one egg per fruit and where only a surviving egg can cause the fruit to abort (Campos, 2001). In these scenarios, the ability to perform effective searching to increase the encounter probability is of an utmost importance. Consequently, behavioral movement parameters such as velocity, time spent in motion and distance covered have a relevant influence in the searching efficacy which is regarded as a key parameter in the potential of natural enemies to exert effective biological control of insects pests (van Roermund et al., 1997).

In behavioral research, automated observation provides significant advantages. Video tracking is a particularly suitable tool to measure movement behavior related to a spatial scale (distance, speed, etc.), providing valuable data that otherwise would be impossible to record accurately (Noldus et al., 2001; 2002; Spruijt et al., 1992). The EthoVision integrated video tracking systems has been used successfully for recording the activity, movement and interactions of several natural enemies such as coccinelid beetles, parasitic wasps, and predatory mites (Drost et al., 2000; Krips et al.,

1999; Ruzicka and Zemek, 2008). In the present study we describe a laboratory methodology set up using EthoVision XT, with the objective of characterizing *Chrysoperla carnea* larval behavior using precisely calculated movement parameters.

4.3 Materials and methods

4.3.1 Insects

A stock colony has been maintained at the Estación Experimental del Zaidín since 2005 from larvae supplied by Koppert Spain (La Mojonera, Almería). On arrival, larvae were individually transferred to Petri dishes and reared on eggs of *Ephestia kuehniella* Zeller (Lepidoptera, Pyralidae) purchased from Biotop (Valbonne, France). After emergence, adults were placed inside plastic rearing boxes (approximately 100 specimens per box) with access to mineral water and artificial diet consisting of 50% honey and 50% pollen spread over filter paper and sprinkled with quartz grains to ease adult lacewings movement on the surface. The food and water were replenished weekly. The stock colony was maintained in a controlled environment cabinet at 25 ± 1°C, 50–60% RH, and a photoperiod of 16:8 (L:D) h. and monthly renewed with additional larvae from the supplier. Experimental individuals of *C. carnea* used in the study were obtained from eggs laid on an ovipositional surface stuck upside-down onto the removable lid of the rearing box and developed using the same culture conditions.

C. carnea individuals chosen for the larval movement video tracking experiment were recently molted third instar larvae of approximately the same length (4–6 mm) and weight (2–4 mg) corresponding to an age of 6 to 7 days old. The predators were starved 24h before the experiment with the objective of guaranteeing mobility by enhancing prey searching activity as it is a stepwise function of the prey deprivation time (Sengonça et al., 1995). *Sitobium avenae* Fabricius (Hemiptera, Aphidae) used in this study were supplied by Koppert Spain (La Mojonera, Almería) and maintained on potted

barley (*Hordeum vulgare* L.) at the same environmental conditions as the predator. Only second and third instar aphids were used in the trials.

4.3.2 Larval movement video tracking

The movement variables were recorded in a 4.5 cm plastic Petri dish covered with its lid. Three types of food sources were offered to *C. carnea* larvae as treatments: (1) a honey drop, (2) *E. kuehniella* eggs and (3) three or four *S. avenae* aphids. In each case, the food sources were situated within an approximately 2 mm diameter area in the exact centre of both the bottom and top of the Petri dish. This set up allowed the movement of the predator on the dish and lid alike with the same influence of the food source. Alive aphids were immobilized preventing them from walking out from the feeding point. In addition, one treatment was without food source, recording the movement variables without the interference of a feeding behavior.

The Petri dishes were placed under the video camera acting as the arena as shown in Fig. 4.1. The larvae were gently transferred to the Petri dish using a camel paintbrush placing it near the border at the maximum distance possible from the food source. As a result of the previously described manipulation, the larvae frequently remained still for several minutes, and therefore the experiment was initiated the moment the predator had calmed down and started to move again. The Petri dish and the food source were replaced for each individual observation.

All the experiments were conducted in a windowless controlled room at 22 ± 2°C and 35 ± 10% RH. Several attempts, with different light intensities at different distances provided by fluorescent tubes and reading lamps, were made in order to achieve a dim light intensity as well as an adequate detection of the predator. A dim light condition was preferred since *C. carnea* larvae have been described to have mainly a crepuscular activity (Canard, 2001). However the larvae appeared to have active movement under intense light conditions. The main problem was inadequate detection due to the

transparency of the insect resulting in a poor contrast with the background and a constant interference of the reflections produced in the borders of the arena. This difficulty was solved by illuminating from the underside with a DCR III fiber optic illuminator (Schott, Elmsford, NY, USA) alone. Contrary to the top illumination, it produced no reflections and it could be situated underneath the arena, in direct contact with it (Fig. 4.1), since it generated no temperature increase. Furthermore, it allowed a reduced light intensity at 130 lux measured in the arena.

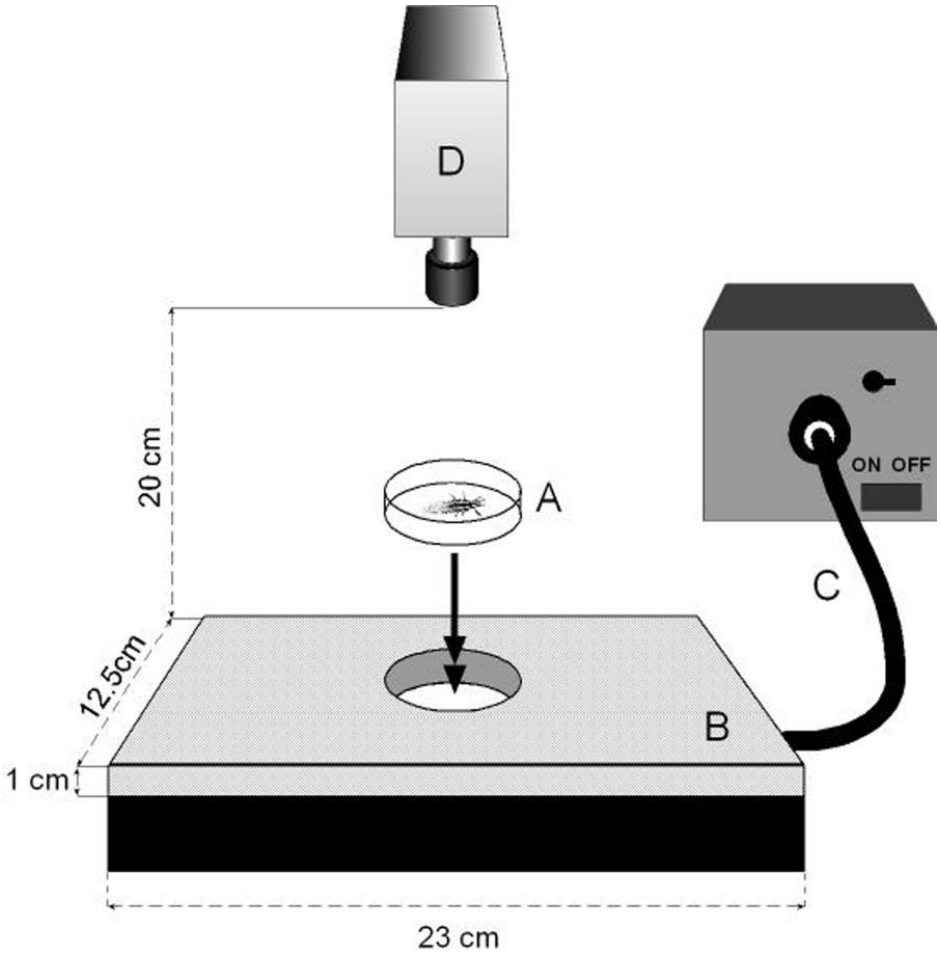

Figure 4.1. Larval movement video tracking experiment setup. (A) experimental arena, (B) arena adjusting corkboard (C) underside illuminator (D) video camera.

The chrysopid movements were analyzed by means of the computerized video tracking system EthoVision (Noldus Information Technology, Wageningen, The Netherlands). Tracks were recorded using a Panasonic CCTV videocamera (Mastsushita Electric Industrial Co., Ltd., Japan) equipped with a

zoom lens and fixed to an arm above the arena (Fig. 4.1). The image signal from the camera was transferred to a video system that included a monitor (Panasonic WV-BM1410 14") and a personal computer with a frame grabber as part of the EthoVision XT system. The image was transformed into a digital signal that could be interpreted by the EthoVision XT software installed in the computer. The grey scale method and a dilation filter were selected in the program settings for object detection of the insect. This method proved to be the most suitable under the illumination conditions previously described and given the translucent nature of the object that was monitored. The detection settings were also adjusted to improve the detection as much as possible. These settings remained fixed throughout the experiments, improving the detection when required in individual trials by slight adjustments of the light intensity allowed by the fiber optic illuminator. The recording was done at the highest possible sample rate, 5.0 frames per second, guaranteeing the most accurate path representation attainable. In the arena definition settings, the food source point and a 15 mm diameter circular feeding zone around it were defined, as a measure of the area of influence of the feeding behavior. The rest of EthoVision settings were left as default.

One individual was tested at a time for a period of 15 min and each larva was used only once. All experiments were performed between 9:00 and 14:00 with a variable number of tests per day depending on the availability of larvae up to a maximum of 10 recordings. Treatments were tested in a random order until reaching a complete set of 25 valid tracks per treatment. Whenever the larvae failed to move in the arena for a period of 10 min before recording, it was slightly touched with a camel paintbrush to check for normal mobility. If after the contact the larvae remained immobile, or abnormally clumsy slow movements were observed, the individual was discarded. If the individual reacted positively, it was set aside for a later trial. After a complete set of daily recordings, the tracks were visualized using the program function “Visualize data” in order to detect anomalous behavior. This typically consisted on a lack of activity not detected previously, characterized by a start of the motion, triggering the trial start, and a cease of the movement occurring within seconds afterwards. In this case the complete track was discarded.

4.3.3 Data analysis

Each digitalized data file was analyzed individually as a replicate extracting a different set of behavioral variables from the tracks in each experiment. A complete description of the variables used herein and the algorithms employed in their calculations are given in Noldus Information Technology (2007). A minimum distance moved threshold of 0.3 cm was established in order to avoid parameter miscalculations derived from a wobbling of the central reference point of the object (Socha and Zemek, 2003). In the larval movement experiment the variables extracted belonged to the two different areas electronically defined. The variables calculated for the entire arena were:

(1) Total distance moved (cm), (2) mean velocity (mm/s), (3) mean angular velocity (unsigned degrees/s), (4) meander (unsigned degrees/cm), (5) movement (%), (6) mean distance to the food source (cm) and (7) time spent in the food source influence zone (s). The variables extracted from the food influence zone alone were: (1) Total distance moved (2) mean velocity (3) mean angular velocity, (4) meander and (5) movement. Angular velocity and meander represent the change in direction in the larva movement. The movement variable was programmed as a measure of the fraction of the total time that the larva spent in motion. The start velocity, that signals the onset of movement, was set at 0.30 mm/s and the stop velocity, below which the system cease to record movement, despite wobbles of the object's center, was set at 0.20 mm/s. These values were established taking into account the average velocity of the individuals recorded in the complete set of larval movement experiments.

The EthoVision output files were imported to the statistical package SPSS 18.0 (SPSS Inc.). Means were calculated for each parameter, treatment and zone, and statistically compared using the Kruskal-Wallis test followed by Mann-Whitney U tests for paired comparisons with α recalculated using

Holm's sequential Bonferroni adjustment (Holm, 1979). All statistical tests were performed at a threshold significance level of 0.05.

In addition, the two data sets derived from the arena and the food influence zone, were subjected to both Principal Component Analysis (PCA) based on the correlation matrixes (so-called scaling 2) to explore the relation between the behavioral variables and the possible membership of tracks represented visually as data clusters (Ramette, 2007). Before choosing the PCA both data sets were checked for a linear response of variables by running a Detrended Correspondence Analysis (DCA), obtaining lengths of gradient below 1 in both cases (Lepš and Šmilauer, 2003). Before analysis the data was centered and standardized.

4.4 Results

4.4.1 Arena

Larvae subjected to trials without the presence of a food source showed more than fivefold higher mean velocity in movement (Kruskal-Wallis, F = 42.17, df = 3, P < 0.01) in relation to the rest of the treatments, and an accordingly higher total distance moved (Kruskal-Wallis, F = 40.81, df = 3, P < 0.01) (Table 4.1). Control larvae moved almost the whole trial time whilst in the rest of the treatments the larvae exhibited stops accounting for approximately a half of the total trial time (Kruskal-Wallis, F = 41.65, df = 3, P < 0.01). The path shape parameters angular velocity (Kruskal-Wallis, F = 46.02, df = 3, P < 0.01) and meander (Kruskal-Wallis, F = 36.99, df = 3, P < 0.001) also differed greatly in control larvae whereas no differences were observed among treatments with food presence. Finally, the fraction of time spent on the food source zone was about a 1% in the control whilst in food presence the larvae spent a higher time (Kruskal-Wallis, F = 30.33, df = 3, P < 0.001), with a maximum time spent in presence of *E. kuehniella* eggs and the minimum in the honey treatment. However no significant differences were found among them. Similar results were recorded for the mean distance to the food source (Kruskal-Wallis, F = 42.17, P < 0.001) that also denotes the food

influence (Table 4.1). The PCA revealed through the correlation matrix that all the parameters within the analysis were correlated (Pearson's correlation, $P < 0.01$) (Fig. 4.2). Extremely high correlation values were obtained for mean angular velocity and meander (correlation coefficient = 0.985, $P < 0.01$), distance to point and time in zone (correlation coefficient = - 0.946, $P < 0.010$) and total distance moved and mean velocity (correlation coefficient = 0.999, $P < 0.001$). The PCA biplot representing graphically the position of the tracks as related to the variables made possible to identify as a differentiated class the tracks belonging to the control treatment (Fig. 4.2).

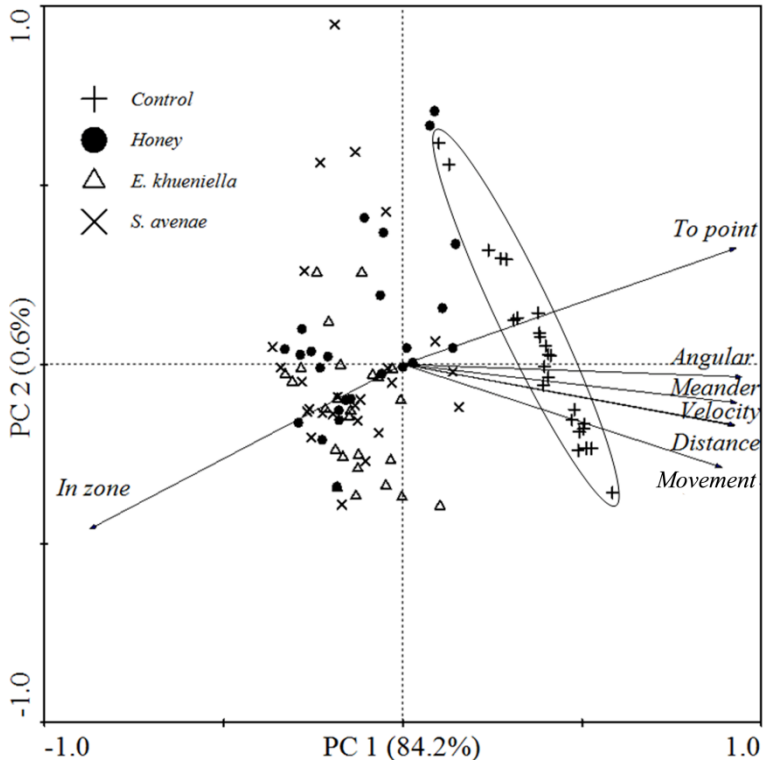

Figure 4.2. Principal components biplot corresponding to the whole arena showing the individual trials (symbols) and the calculated variables (arrows). The variables represented are: Total distance moved (Distance), mean velocity (Velocity), mean angular velocity (Angular), meander, movement, mean distance to the food source (To point) and time spent in the food source influence zone (In zone). A circle has been drawn around the only visually identifiable class.

Table 4.1. Behavioral parameters obtained from Ethovision XT for the larval movement experiment for the arena and the feeding zone.

Area	Parameter	Treatment [a]							
		Control		Honey		*E. kuehniella*		*S. avenae*	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Arena	Distance moved (cm)	477.37	194.16 a	75.79	46.73 b	61.98	67.12 b	67.36	54.67 b
	Velocity (mm/s)	5.31	2.16 a	0.87	5.51 b	0.70	0.75 b	0.75	0.60 b
	Angular velocity	37.09	46.66 a	284.15	108.83 b	298.48	92.25 b	316.66	123.82 b
	Meander (degrees/cm)	775.42	1035.26 a	6288.00	2570.95 b	6144.92	2182.61 b	6808.65	2966.46 b
	Movement (%)	90.15	13.08 a	51.90	14.79 b	57.21	12.93 b	49.88	16.12 b
	Distance to point (cm)	2.36	0.15 a	1.00	0.62 b	0.66	0.27 b	0.77	0.42 b
	Duration in zone (%)	1.13	0.79 a	65.26	30.81 b	81.22	17.91 b	70.70	28.87 b
Food zone	Distance moved (cm)	9.03	5.48 a	15.39	11.64 b	22.84	8.04 c	23.00	13.11 bc
	Velocity (mm/s)	10.15	3.22 a	0.51	1.10 b	0.34	0.16 b	0.53	0.79 b
	Angular velocity	15.09	48.65 a	427.29	93.90 b	340.52	80.16 c	357.10	141.00 bc
	Meander (degrees/cm)	290.57	1116.13 a	9533.66	2750.78 b	6979.44	2026.87 c	7820.73	3047.73
	Movement (%)	1.06	0.71 a	26.43	30.81 b	43.40	13.09 c	32.14	16.43 bc

[a] Values within the same row followed by different letter(s) are significantly different (Mann-Whitney *U*-test, Bonferroni-Holms correction $\alpha < 0.05$).

4.4.2 Food source zone

As in the arena, the velocity showed by the larvae in the food source zone was higher without the presence of food (Kruskal-Wallis, $F = 48.91$, df = 3, $P < 0.001$) with no difference among the rest of treatments (Table 4.1). However, the total distance moved within the marked zone was significantly higher when offered *E. kuehniella* eggs (Kruskal-Wallis, $F = 42.20$, df = 3, $P < 0.001$) than when offered honey or aphids. The same trend was observed when looking at the parameter movement, with a different meaning than the same parameter measured on for the entire arena, as it expresses the fraction of the total time that the larvae spent moving on the marked area. The highest value of the parameter movement corresponded to the *E. kuehniella* eggs (Kruskal-Wallis, $F = 48.69$, df = 3, $P < 0.001$) treatment and the lowest to the control. In this case, the movement value in the feeding zone was significantly lower in presence of a honey drop (Table 4.1). Path shape parameters also differed among treatments coincidentally, higher values were recorded in the control whilst significantly lower values in presence of honey (angular velocity, Kruskal-Wallis, $F = 49.58$, df = 3, $P < 0.001$; meander, Kruskal-Wallis, $F = 49.23$, df = 3, $P < 0.001$). The correlation analysis indicated a similar relationship among variables as for the entire arena with the exception of mean velocity and distance moved that did not correlate, (correlation coefficient = -0.429, $P = 0.117$). The rest of variables recorded showed significant correlation (Pearson's correlation, $P < 0.05$). As above, the concordance between the path shape parameters was very strong (correlation coefficient = 0.969, $P < 0.01$). The PCA biplot diagram (Fig. 4.3) summarizes the correspondence between variables, and as before, it allowed differentiating the class corresponding to the control treatment.

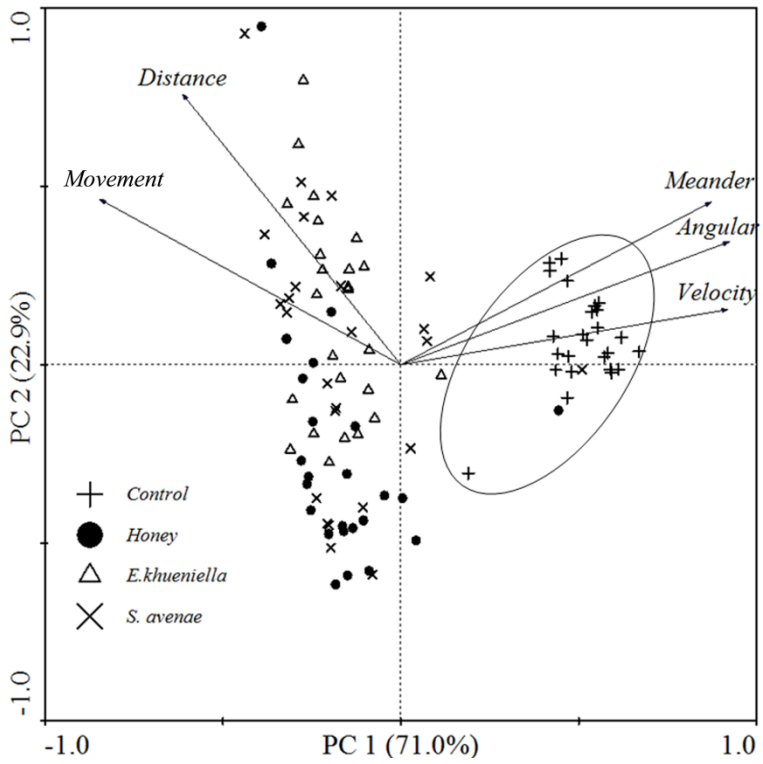

Figure 4.3. Principal components biplot corresponding to the feeding zone showing the individual trials (symbols) and the calculated variables (arrows). The variables represented are: Total distance moved (Distance), mean velocity (Velocity), mean angular velocity (Angular), meander and movement. A circle has been drawn around the only visually identifiable class.

4.5 Discussion

A complete and varied set of displacement related variables were analyzed in order to typify the lacewing larvae behavior in as much detailed as possible. The record of the variables in different zones further confirms the existence of completely differential behavioral pattern of movement in the presence of any kind of food in opposition to a blank arena. With food present, lacewing larvae spent more time in the feeding zone. Later observation of the tracks revealed that in some trials they headed directly to the food source, in others it took a bit longer. But always eventually, as exhibited by the recorded variables, they found the source and spend an important amount of time exploring and consuming the prey.

The parameters indicating the time in motion and the total distance covered in the feeding area also supported this observation, and the fact that, during prey manipulation, and furthermore, with several preys clustering, displacement and lifting of prey as well as twisting and turning movements are usual (Principi and Canard, 1984). The comparison between walking speed on the zone in the presence and absence of food confirmed that this locomotion, associated to food manipulation, was always slower than that derived from a prey searching behavior, where the larvae raced across the zone swiftly. Several variables extracted from the movement in the whole arena (Fig. 4.2) suggest an intense searching activity performed by larvae in absence of food. Thus, these larvae covered a far greater distance at an increased speed and remained in motion for almost the total length of the trial. The reduced activity in the rest of treatments as shown by the most of the variables can be explained by the feeding action itself, once the prey has been encountered, and the inactive state originate as a result of the food consumption. These behavioral features clearly position the tracks as referred to the measured variables (Figs. 4.2, 4.3) in two groups or classes, the control treatment, and the rest. Honey, included in the category of the food sources is not prey and hence, not an appropriate aliment. The multivariate analysis failed to classify a behavioral response to this treatment based on the movement variables recorded. Nonetheless, a decreased activity on the feeding zone in presence of a honey drop in terms of distance moved and time spent moving was observed (Table 4.1). Besides, the time spent on the feeding zone was lower compared to the time spent in real prey presence, although this value was not significantly different. The results indicate that the larvae employed a considerable amount of time close to the honey drop, and that the absence of any other kind of suitable prey is likely to have induced the larva, in a starvation situation, to try repeatedly to feed on the drop for a long period of time. However, this behavior lacked the active movements that characterize natural prey handling that were typical during the consumption of *E. kuehniella* eggs and aphids. Without stimuli (control treatment) larvae moved at a very high speed and kept themselves away from the central zone of the arena. Tracks pattern observation revealed that the insect showed a tendency to move circularly around the Petri dish border. This behavior possibly responds to the existence of artificial boundary in the arena, eliciting the natural *C. carnea* trend to

follow morphological guidelines as edges in searching for their prey (Arzet, 1973). This swift and regular movement was not shown by larvae with access to food, which, as mentioned above, decreased their activity.

The development of the EthoVision bioassay for lacewing larvae opens new possibilities in the study of this valuable predator. Recent methodological applications in biological control research range from behavioral response to chemical cues (Ruzicka and Zemek, 2008; Stewart-Jones et al., 2006) to searching efficiency (Drost et al., 2000). Beneficial arthropods effects on mobility as a result of insecticides exposure are not often directly studied. Mobility data is not accurately measured with quantitative variables preventing the use of statistical analysis (Desneux et al., 2007). EthoVision bioassay allows recording these variables precisely with the capability of detecting even small differences in the behavioral response of *C. carnea*. Apart from the already mentioned applications the automated tracking system is suitable to assess the impact of pest control compounds on the mobility of this beneficial species so frequent in laboratory testing.

Acknowledgements

The authors would like to thank Dr. F. Ruano for her advice and assistance, Dr. B. Cotes for her critical reading of the article and suggestions and H. Barroso for rearing the insects. This work was conducted with funding from the Junta de Andalucía (grant P07-AGR-02747).

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5. Biological and behavioral effects of kaolin particle film on larvae and adults of *Chrysoperla carnea* (Neuroptera: Chrysopidae)

Biological and behavioral effects of kaolin particle film on larvae and adults of *Chrysoperla carnea* (Neuroptera: Chrysopidae)

Published in *Biological Control*, Volumen 59, Issue 2, pages 98-105, July 2011

5.1 Abstract

Several laboratory and field experiments were conducted to investigate the effect of kaolin particle film formulation Surround WP on the biology and behavior of the common generalist predator *Chrysoperla carnea* (Stephens). Kaolin 5% (w/v) suspension direct spray did not affect third instar larvae development to adulthood. The hatchability of recently laid eggs, subjected to the same spraying process, was also unaltered. However, third instar larvae coated with particle film after kaolin spraying showed slightly hampered movement capacity after measuring : distance moved, mean velocity, angular velocity and time spent in motion, obtained using the computerized system EthoVision. Parameters extracted from recorded larvae movement on a kaolin film surface showed similar decreased mobility results as well as preference for the clean control surface. Additionally, the larvae had difficulty grasping treated leaves. *C. carnea* adult females showed a predominant preference for treated leaves in oviposition choice tests. In the field trial, no difference in *C. carnea* adult abundance was found between kaolin-treated and control olive trees. These results indicate that disruption of movement capacity and dislodgement from the plant surface may be the principal negative effects of particle film on *C. carnea* larvae. Despite the positive trend in oviposition towards kaolin treated surfaces, a particle film attraction effect on adults was not observed at field level.

Keywords: EthoVision; green lacewing; natural enemies; olive; predator; video tracking

5.2. Introduction

Kaolin particle technology is a relatively new and promising option for the reduction of pest and disease damage in certain crops. The plants are dusted or sprayed with particles of this non-abrasive, chemically inert, aluminosilicate [$Al_4SI_4(OH)_{18}$] mineral, creating a film that coats the plant and acts as a protective barrier against both pathogens and pest arthropods (Glenn et al., 1999). The use of kaolin as a pest management strategy has proved effective as a broad spectrum compound against a wide range of pest insects such as the psyllid *Cacopsylla pyricola* Foerster on pear, *Diaphorina citri* Kuwayama on citrus (Hall et al., 2007; Puterka et al., 2005), the codling moth *Cydia pomonella* (L.) on pear (Unruh et al., 2000), Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) on apple (Mazor and Erez, 2004), the thrip *Thrips tabaci* Lindeman on onions (Larentzaki et al., 2008) and the aphid *Myzus persicae* (Sulzer) on peach (Karagounis et al., 2006). Specifically, in olive farming, recent research has shown particle film effectiveness in suppressing the olive fruit fly *Bactrocera oleae* Gmel., the key pest in this crop (Pascual et al., 2010; Saour and Makee, 2004) and the black scale *Saissetia oleae* (Olivier) (Pascual et al., 2010). These observations point to the feasibility of kaolin particle films as a viable alternative to extensively using insecticidal control. Particle film technology also has the advantage of being permitted in organic agriculture, which, alongside mass-trapping (Porcel et al., 2009) and naturally derived pesticides (Iannotta et al., 2007), is one of the few options available to organic olive growers to control *B. oleae* damage.

Nevertheless, pest control methods that inhibit the action of harmful insects need to be evaluated for their effects on beneficial arthropods in order to increase our knowledge of the possible impact caused by their application. This knowledge is useful in joint strategies where alternative pest control methods should not interfere with biological control. Recent literature has thus generally focused on field assessment of kaolin effects based on the presence or absence of beneficial arthropods on kaolin-treated crops (Karagounis et al., 2006; Marko et al., 2008; Pascual et al., 2010; Sackett et al., 2007). The mechanisms underlying how particle films affect the biology and behavior of insect pests have been extensively explored by several authors

(Cadogan and Scharbach, 2005; Lemoyne et al., 2008; Puterka et al., 2005), who describe a variety of effects: direct toxicity and interference with insects' ability to settle, move or oviposit (Hall et al., 2007). However, to the best of our knowledge, the specific effects of kaolin films on beneficial insects have been the subject of limited study, and in the case of predators, no information on possible behavioral and biological disruptions is available.

The green lacewing *Chrysoperla carnea s. lat.* (Stephens) (Neuroptera: Chrysopidae) is one of the most common, naturally occurring, arthropod predators (Duelli, 2001). Its extensive range of prey, including almost all soft-bodied arthropods, its wide distribution, as well as its voracious feeding capacity, make this natural enemy a promising candidate for pest management programs (Tauber et al., 2000). Due to its overall importance as a foliage-dwelling entomophagous predator, this insect has been regarded as an appropriate test species for the assessment of novel pest management compounds (Mandour, 2009; Medina et al., 2003). In olive orchards, the green lacewing is considered the major oophagous predator of the olive moth *Prays oleae* Bernard, helping to reduce the economic impact of this pest. It is also known to prey upon less harmful insects such as the black scale and the olive psylla *Euphyllura olivina* Costa (Campos, 2001). Studies undertaken on different crops have detected decreased abundance and alteration of the assemblages of polyphagous predators associated with kaolin treatments (Marko et al., 2008; Pascual et al., 2010; Sackett et al., 2007). Therefore, the aim of the present study is to evaluate both the biological and behavioral effects of kaolin particle films on *C. carnea* predator.

5.3. Materials and methods

The kaolin clay-based particle film used in the present study was the hydrophilic Surround WP crop protectant manufactured by NovaSource (Salt Lake City, UT, USA). The compound is based on processed hydrous kaolin particles (1.0 ± 0.5 μm ø), with an incorporated synthetic hydrocarbon spreader-sticker that enhances particle adhesion to the plant. The compound, hereafter referred to as kaolin, was applied at a rate of 5% in mineral water (50

gr/L) as recommended by the manufacturer. Plain mineral water was used as control, and both were applied in all laboratory experiments using an airbrush spray gun (Model 350, Badger Air-Brush Co., Franklin Park, IL, USA) connected to an air compressor generating a cone spray pattern. The spray gun was fixed by means of an adjustable height laboratory arm at 33 cm over the table surface, preventing excessive air flow over the spraying spot position. The spray gun bottle was constantly in contact with a magnetic stirrer held by the same arm in order to avoid kaolin particle sedimentation during the spraying process.

In all laboratory experiments, with the exception of the experiment described in Section 2.3, surfaces and the larvae themselves were sprayed for a 30 s period. To quantitate kaolin particle retention, preweighed Teflon slides (97 cm^2) were similarly sprayed, and, after a 4 h drying process at 25 ºC, were weighed again using an electronic analytical balance (AS 220/C/2, RADWAG Balances and Scales, Radom, Poland). The difference between final and initial weights enabled us to calculate the weight of the kaolin film deposited on each slide. The process was replicated up to 20 times. Finally, mean kaolin film weight was divided by the total surface to obtain the deposit per surface unit ($\mu g/cm^2$).

5.3.1. Rearing C. carnea in the laboratory

The larvae used in all experiments were obtained from *C. carnea* eggs collected from a stock colony established in 2005 with larvae supplied by Koppert Spain (La Mojonera, Almería) and were renewed monthly. Larvae were individually reared in Petri dishes and fed on eggs of *Ephestia kuehniella* Zeller (Lepidoptera, Pyralidae). Adults were kept in boxes with an ovipositing surface and provided with an artificial diet (50% honey and 50% pollen) and mineral water. Both were maintained in a controlled environment cabinet at 25 ± 1 ºC, 50–60% RH and a photoperiod of 16:8 (L:D) h.

5.3.2. Acute mortality to larvae

C.carnea larvae were directly sprayed with the 5% kaolin suspension in order to assess any possible effects on mortality**.** A sufficient number of newly laid eggs were removed from the adult-rearing boxes and developed in the same culture conditions as described above. Newly molted third instar larvae were chosen for the experiment given that previous observations had revealed that molting caused effective removal of the particle layer deposited on the larva's dorsal cuticle when spraying first and second instars. The fact that third instar larvae necessarily undergo both cocoon spinning and metamorphosis with a kaolin film attached makes them a priori more susceptible. The inhibition of these developmental processes, whatever its origin, leads in most cases to the individuals' decease (Cadogan and Scharbach, 2005; Liu and Chen, 2001; Vogt et al., 2001). Recently molted larvae (less than 12 h), homogeneous in age and size, were selected and set aside from the stock. Individuals were transferred by gently tapping the underside of the Petri dish or, when necessary, by using a camel paintbrush, to 4.5 cm ø Petri dishes containing a double filter paper layer in order to prevent the formation of large droplets during the spraying process. Larvae were chilled for 15 min, after which they were sprayed with either kaolin suspension or water. After spraying, the thoroughly wetted larvae typically presented the formation of one or more droplets on the insects' dorsum. The Petri dish was then covered, and the individuals were subjected to a 1 h drying period, after which, in the case of kaolin-sprayed larvae, the particle film became visually apparent. Finally, the filter paper was removed, and the larvae were fed and kept in culture conditions. The experiment used a randomized complete block design (RCBD), with replications consisting of 10 insects per treatment replicated five times over time. Tested individuals were checked daily for survival up to adult emergence and the developmental stage in which mortality occurred was recorded.

5.3.3. Acute mortality to eggs

Kaolin film applications were tested on *C. carnea* eggs to determine their effects on egg viability and larval hatching suppression. Two bands (17 × 9 cm) of self-adhesive green velvety paper (Sadipal Stationery Papers, Girona, Spain), used as ovipositing surfaces in the *C. carnea* stock colony, were attached upside-down to the rectangular removable lid (36 × 22 cm) of the adults' rearing box containing approximately 100 mixed male and female adults. After 12 h, 30 eggs were selected on each band by discarding the rest. One band was sprayed with the kaolin suspension and the other with the control water. Both treatments were delivered by hand-holding the airbrush at an approximate distance of 10 cm and by spraying the whole band area for 30 s. The eggs were observed under the microscope (mag) to detect small droplets of kaolin suspension. After drying, the suspension droplets produced fragments of particle film adhering to the egg's surface. The lid containing the two treated sets of eggs was kept in a cabinet under culture conditions. After three days, *E. kuehniella* eggs were sprinkled over the bands in order to feed neonate larvae to prevent intraspecific egg predation. From the fourth day onwards, newly hatched *C. carnea* larvae were removed daily from the bands by means of a camel paintbrush, and the eggs were checked daily for 8 d to ensure that hatching had ceased. Data regarding viable and non-viable eggs was recorded under a microscope. The non-viable eggs were identified as those that desiccated as well as neonate larvae that failed to free themselves from the eggshell and died in the process. The experimental design used was a RCBD, with up to 10 replications being carried out over time.

5.3.4. Effect on leaf-grasping ability

Larvae's capacity to grasp leaf surfaces covered by kaolin particle film as compared to untreated leaves was assessed. Olive leaves (cv. Picual) were collected, cut, and attached side by side to a 100 cm^2 square glass, forming a continuous leaf surface platform. Several platforms were created as described, using either the upper or lower side of the leaves for each platform. Leaf-covered platforms were pressed by means of a weight and kept at cold

temperatures to avoid moisture loss. Following standard spraying methodology, the platforms were firstly sprayed with water and left to dry for 2 h. Newly molted (less than 12 h) third instar individuals were raised and chosen as described in Section 2.2. The experimental methodology adopted to assess differential grasping ability is similar to that described for psyllids by Puterka et al. (2005). Leaf platforms were horizontally mounted on a laboratory arm that enabled them to rotate 180º. The larvae were transferred to the leaf surface and allowed to move for several seconds. When the larva was located in the central part of a leaf (sufficiently away from the edge) and still in motion, the platform was inverted for 30 s to determine whether the larvae could continue gripping the surface or falls off the platform. This procedure was repeated using 10 different larvae, after which the platform was sprayed with kaolin, left to dry and used to assay 10 new larvae on the particle film. The whole process was repeated using the underside of the leaf surface. The experiment was carried out using RCBD and was replicated five times over time. Two new leaf platforms (upper and lower surfaces) were constructed per replication.

5.3.5. Effect of particle film covering C. carnea larvae on mobility and behavior

Directly sprayed kaolin-covered larvae were assessed for changes in behavioral and locomotor parameters. The same size and age of third instar larvae were selected as in previous experiments, which were sprayed with either kaolin or mineral water. Once sprayed, the larvae were starved for a period of 24 h prior to bioassay. Each individual was moved to a windowless controlled room (22 ± 2 °C, 35 ± 10% RH and 130 lux light intensity), placed in a 4.5 cm Petri dish and viewed with a Panasonic CCTV video camera (Mastsushita Electric Industrial Co., Ltd., Japan). Larval movement was recorded for a period of 15 min, and the track was transferred to a computer as part of the EthoVision XT integrated video tracking system (Noldus Information Technology, Wageningen, The Netherlands). The EthoVision software automatically determines the location of the individual larva in the area and calculates several movement parameters derived from changes in position. The parameters chosen were (1) total distance moved (cm), (2) mean

velocity (mm/s), (3) mean angular velocity (degrees/cm) and (4) movement (%). The movement variable was defined as the fraction of time the larvae spent in motion. Parameter descriptions are given in Noldus et al. (2002) and algorithms and calculations are described by Noldus Information Technology (2007). One individual at a time was tested, and each larva was used only once. The experiment was conducted on the basis of a completely randomized design (CRD) over five consecutive days, running 10 to 15 trials of both treatments every day up to a total number of 30 valid replicates per treatment (60 replicates).

5.3.6. Effect of particle film surface on larval mobility and selection

In this experiment, we determined whether kaolin-covered substrates interfered with larval mobility parameters and whether they showed a substrate preference between treated and control surfaces. As in previous experiments, newly molted third instars were obtained from eggs selected for this purpose. The larvae were subjected to a 24 h starvation period before bioassay. The 4.5 cm Petri dish experimental arena was divided into two surface halves of equal size. Following the usual methodology, one half was sprayed with kaolin and the other half with the control water. A semicircular waterproof plastic was used to cover treatment areas during the spraying process, ensuring that they never came in contact with the particle film, as the treatment was always delivered after the control area had been sprayed and dried for 2 h. Given that only the lower part of the Petri dish was sprayed, the experimental design was restricted to this area by coating the dish's circular edge with Fluon (AGC Chemicals America Inc., Moorestown, NJ, USA), thus preventing the larva from climbing up to the lid. The experimental setup and procedures were similar to those described in Section 2.5. The parameters extracted from the recorded tracks were (1) total distance moved (cm), (2) mean velocity (mm/s), (3) mean angular velocity (degrees/cm), (4) movement (%) and (5) time spent in each zone (kaolin and control). The parameters were calculated for each zone individually for the purpose of comparison. Unlike the previous experiment, each individual trial generated the complete set of variables for both treatments. The experiment was carried out over five consecutive days for a total of 50 replicates. Each block of replicates consisted

of 10 trials conducted on the same day with the same experimental arena, and a new arena was therefore sprayed each day.

5.3.7. Adult oviposition preference

C. carnea adults were placed in an oviposition arena and given the choice between kaolin film and control surfaces. The arena consisted of an 8.5 cm ø × 2.5 cm high Petri dish with two ovipositing leaf surfaces, one upside down attached to the lid and the other attached to the bottom of the Petri dish. The 8.5 cm ø surfaces were made as described in Section 2.4. Upper and lower surfaces were prepared per arena. Before mounting the surfaces on the arena, both were divided into equal semicircular zones and sprayed with kaolin and water, proceeding, as described in Section 2.6, by means of a larger plastic cover. As a result, both leaf surfaces contained particle film and control zones of equal area. The upper surface adhered to the bottom of the dish, and the lower surface to the dish's lid. The lid was placed on the dish so that the kaolin film zones always faced the control zones. Newly emerged (after less than 12 h) adults were coupled and transferred to small rearing boxes (0.9 L), kept in culture environmental conditions and provided with adult food and water. After seven days, individual couples were transferred to the ovipositing arena to lay eggs for a period of 48 h. Water, supplied by a moistened piece of sponge, and food, supplied directly, were attached to the sides of the dish, while avoiding interference with the surfaces. The eggs deposited in each designated area (upper and lower surfaces, kaolin film and control zones) were recorded. Females that did not oviposit were excluded from the experiment. Ten couples were successively assayed in the same arena, and the process was repeated with a total of five different arenas (50 individual replicates). The experiment used a RCBD design, where each arena constituted a different block.

5.3.8. Effect on adult abundance. Field case study

A field experiment was conducted to determine whether a particle film sprayed on olive trees had an immediate effect on adult lacewing presence. The experiment was conducted in a 258 ha commercial non-irrigated olive

orchard (cv. Picual, 90 yrs old) under integrated pest management (IPM) situated in the province of Granada (37º 17' 46.7" N, 3º 46' 28.7" W), Spain. No insecticidal treatment had been applied during the year, and natural regeneration cover was present between trees from the beginning of spring until natural drying in June. A 50 g/kaolin solution (treatment) and water (control) were applied to 16 trees (grown 11 × 11 m apart) in a square plot by means of a tractor-drawn turbo atomizer (Sistromatic AL-TAR 2000N, Máñez y Lozano S.L. Maquinaria Agrícola, Valencia, Spain) at a rate of 95 L/ha, delivering approximately 0.58 kg of kaolin per tree. Treatment and control plots were situated at a minimum distance of 80 m apart. The experiment was replicated up to four times (total 8 plots) following a RCBD with block separations of at least 150 m. Kaolin was applied twice: on 15th June and 24th Sept 2009. These dates were chosen due to the known chrysopid flying peaks in southern European olive orchards. The chrysopids were sampled seven days after the applications on two consecutive days using an insect aspirator (Modified CDC Backpack Aspirator Model 1412, John W. Hock Co., Gainsville, FL, USA) to sample all the trees in the plot. Inner and outer branches of each olive tree were suctioned up to a height of 2 m for a period of 2 min. To do this, we moved around the tree in order to cover all possible angles. The adult chrysopids collected were counted and identified at species level. Precipitation data obtained from a public agroclimatic station (IFAPA, Junta de Andalucía) revealed a single rainfall event (5.4 mm) that took place the afternoon after the kaolin treatment was carried out on 15th June and three rainfall events between 23rd September and 1st October, adding up to total rainfall of 10.2 mm.

5.3.9. Statistical analysis

All the statistical analysis was carried out using the SPSS Statistics 18 package for Windows (SPSS Inc., Chicago, IL, USA). To analyze larvae and egg acute mortality and larvae leaf-grasping experiments (Sections 2.2, 2.3 and 2.4), percentages were arcsin-transformed for normalization and compared using the Student's *t*-test ($\alpha = 0.05$) for paired comparisons and analysis of variance tests (ANOVA) for multiple comparisons. ANOVA analysis was

followed by Tukey tests (α = 0.05) to identify mean differences. For larval mobility analysis (Sections 2.5 and 2.6), the movement parameter was $\log_{10}$ (x + 0.5), transformed for normalization and compared by means of a Student's t-test (α = 0.05). In all cases, untransformed means are presented. Whenever the data distribution failed to satisfy parametric analysis assumptions, data was subjected to non-parametric Mann–Whitney *U* tests (α = 0.05). Statistically significant differences at a confidence level of α = 0.10 are shown. In the oviposition preference experiment (Section 2.7), as the data set violated parametric analysis assumptions, the treatments were compared using a Kruskal–Wallis test, followed by paired Mann–Whitney *U* tests for individual comparisons. The α value was adjusted by means of the Bonferroni–Holm correction (α ≤ 0.05) (Holm, 1979). Finally, *C. carnea* adult capture data from the field case study (Section 2.9) was analyzed using a generalized linear mixed model (GLMM) with a Poisson error distribution (R software, version 2.1.2, package glmmML). Treatment (kaolin and control) and date were used as explanatory variables and plot as a random effect. No data overdispersion was detected.

5.4. Results

The mean particle density (±SD) deposited on the Teflon slides after drying for 4 h was 290.7 ± 99.7 μg/cm^2.

5.4.1. Acute mortality to larvae

Table 5.1. Mean mortality (± SD) in different development stages and mean adult emergence (± SD) of third instar larvae sprayed either with water (control) or kaolin.

Treatment	% mortality[a]			% adult
	3rd instar	Prepupa	Pupa	emergence[a]
Kaolin	12.0 ± 17.9	4.0 ± 8.9	10.0 ± 7.1	74.0 ± 15.2
Control	14.0 ± 11.4	8.0 ± 10.9	8.0 ± 8.4	70.0 ± 14.1

[a] Data arcsin-transformed for statistical analysis. Mean values (± SD) followed by same or no letters are not significantly different (α = 0.05).

No difference in mortality was observed between the treatment and control individuals (Table 5.1) neither at the different developmental stages (ANOVA, $F = 0.48$, df = 5, $P = 0.788$) nor in the percentage of adult emergence (*t*-test, $t = 0.42$, df = 7.97, $P = 0.685$). The clearly observable kaolin film particle covering the larvae dorsum did not interfere with *C. Carnea*'s normal development from the third instar state.

5.4.2. Acute mortality to eggs and larval survival

Egg hatching and early survival of newly emerged first instars were not affected by the kaolin treatment on eggs under the tested environmental conditions. Specifically, 80.0 ± 8.3% (mean ± SD) particle film sprayed eggs produced normally hatching individuals, while the hatching rate in water sprayed eggs was 84.3 ± 5.9%. Despite a lower hatching rate resulting from the kaolin treatment, no statistical differences were found (*t*-test, $t = 0.36$, df = 18, $P = 0.192$), which can be considered of little biological significance. No cannibalistic egg predation was observed while recording the viable and non-viable eggs, indicating that the recently hatched larvae fed on the *E. kuehniella* eggs supplied.

5.4.3. Effect on leaf-grasping ability

Table 5.2. Mean (± SD) percentage of *C. carnea* larvae able to grasp to kaolin treated and control leaf surfaces and therefore not falling within 30 s after inverting them.

Treatment	% grasping the leaves[a]	
	Upper surface	Lower surface
Kaolin	22 ± 11 a	62 ± 16 b
Control	88 ± 11 c	92 ± 8 c

[a] Data arcsin-transformed for statistical analysis. Mean values (± SD) followed by equal letters are not significantly different (Tukey's test, $\alpha = 0.05$).

Kaolin particle film covering both sides of olive leaves clearly affected *C. carnea* larval ability to grasp the leaf surface. Their grasping capacity was altered by kaolin treatment on both the upper and lower side of the leaf

(ANOVA, $F = 35.8$, df = 3, $P < 0.05$) and was especially notable in the case of the upper side, where the number of larvae that remained on the kaolin-treated surface decreased by 66%. With respect to the upper and lower parts of the leaf, no difference in grasping performance was observed when comparing water sprayed surfaces. However, the ability to grasp the treated lower surface was significantly greater than the capacity to grasp the treated upper surface (Table 5.2).

5.4.4. Effect of particle film covering C. carnea larvae on mobility and behavior

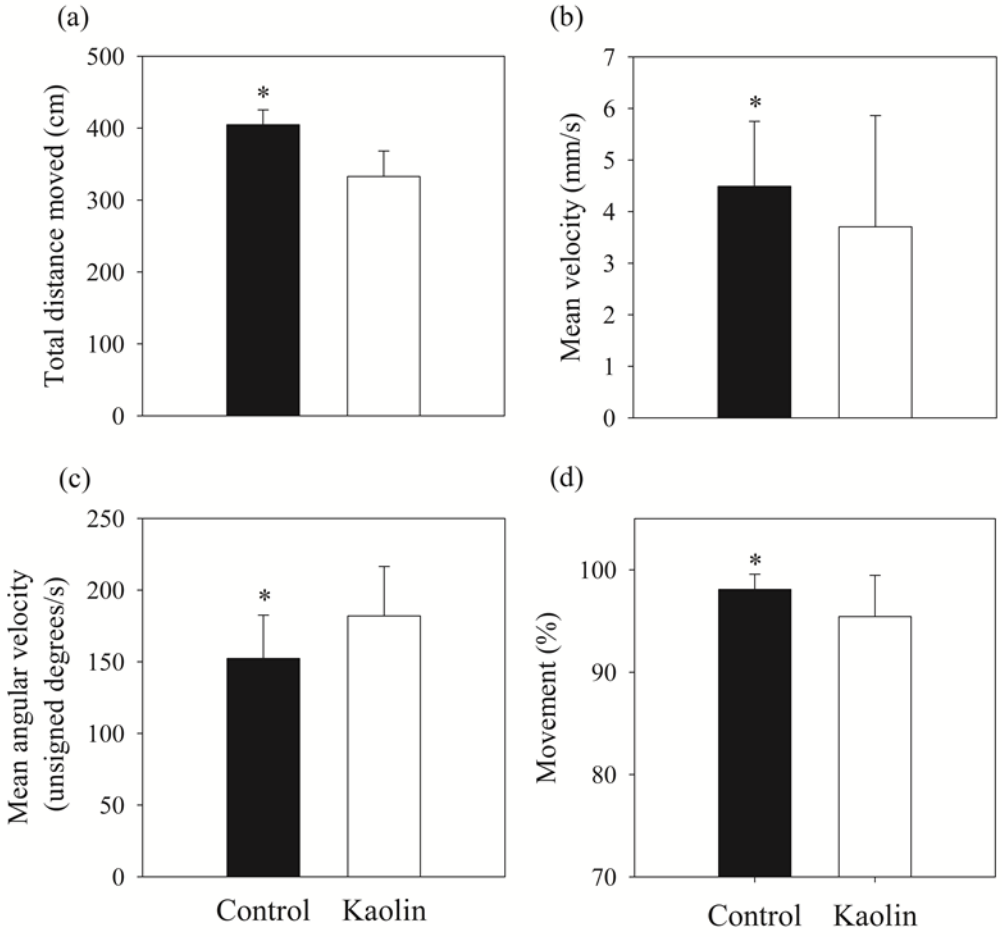

Figure. 5.1. Movement parameters of *C.carnea* larvae directly sprayed either with kaolin or mineral water (control). Mean ± SE (a) total distance moved (cm), (b) mean velocity (mm/s) (c) mean angular velocity (unsigned degrees/s) and (d) movement (%). Bars with different symbols indicate statistically significant differences ($P < 0.05$).

Observation of the recorded tracks did not reveal the existence of a clear behavioral change resulting from the particle film adhering to the insect's surface. Despite this, the numerical variables derived from the tracks provided by the EthoVision XT software showed small but significant differences in all of the measured parameters (Fig. 5.1). Kaolin-treated larvae covered a shorter distance within the arena than water-treated individuals (*t*-test, $t = 2.60$, df = 44.23, $P = 0.012$). The reduction in the distance moved can be interpreted as both an effect produced by a decreased mean velocity of kaolin-treated individuals (*t*-test, $t = 2.56$, df = 44.59, $P = 0.014$) and an increased frequency of pausing or spending a lower fraction of the total trial time in motion (Mann–Whitney *U* test, $Z = –2.61$, $P = 0.009$). Kaolin treatment also affected the shape of the path travelled by the larvae; kaolin-covered individuals showed a significantly higher turning rate per unit of time (*t*-test, $t = 2.56$, df =58.00, $P = 0.001$).

5.4.5. Effect of particle film surface on larval mobility *and choice*

As above, direct observation did not reveal a distinct reaction caused by the presence of a kaolin film. Unlike the results described in Section 3.3, where the larvae moved freely across both the dish and the lid, the individuals showed a recurrent trend to attempt to climb the dish edge from kaolin and control surfaces alike. Analysis of track data showed differences in movement parameters induced by the kaolin film (Fig. 5.2). The tested individuals covered a shorter distance (*t*-test, $t = 2.49$, df = 98.00, $P = 0.014$) at a lower velocity (Mann–Whitney *U* test, $Z = - 1.66$, $P = 0.097$) on the treated surface. The larvae spent significantly less time on the kaolin film surface (*t*-test, $t = 3.78$, df = 98.00, $P = 0.007$) and showed a higher stopping frequency (*t*-test, $t = 3.01$, df = 98.00, $P = 0.007$) as compared to the control surface. No differences were detected in the path shape given that the angular velocity exhibited was similar on both surfaces (*t*-test, $t = 1.05$, df = 82.45, $P = 0.295$).

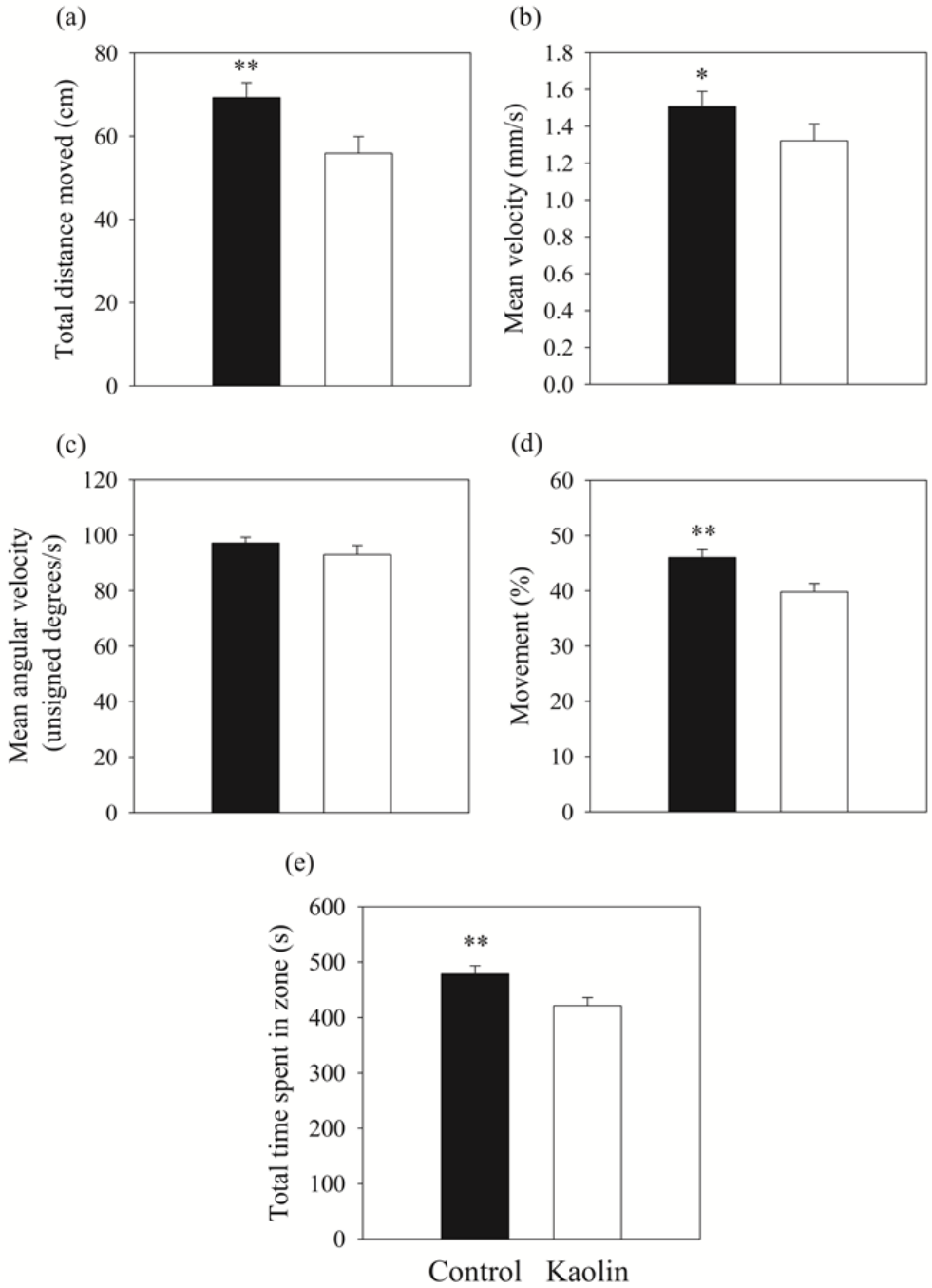

Figure. 5.2. *C. carnea* larvae movement parameters on a circular arena half of which is covered with a kaolin particle film and the other half kaolin free (control). Mean ± SE (a) total distance moved (cm), (b) mean velocity (cm/s) (c) mean angular velocity (unsigned degrees/s), (d) movement (%) and (e) total time spent in zone. Bars with different symbols indicate statistically significant differences ** $P < 0.05$; * $P < 0.10$.

5.4.6. Adult oviposition preference

Table 5.3. Mean number (± SD) of eggs laid by *C. carnea* 7-d-old females in a 48 h time period on upper and lower olive leaves surfaces treated either with kaolin particle film or mineral water (control).

Treatment	# eggs laid [a]		
	Upper surface	Lower surface	Lateral Border
Kaolin	0.2 ± 0.7 a	17.3 ± 8.7 b	1.2 ± 1.7 d
Control	0.0 ± 0.0 a	7.3 ± 6.5 c	

[a] Mean values with different letters indicate statically significant differences (Mann–Whitney *U*-test, Bonferroni-Holm's correction $\alpha \leq 0.05$).

C. carnea female adults laid almost all the eggs upside down on the lower part of the leaves attached to the lid, while just a few were deposited on the lower side and the lateral border (Table 5.3). On the lower surface, where the eggs accounted for 94.9 ± 6.4% (mean ± SD) of the total, the tested individuals laid more than twice the number of eggs on the kaolin-treated semicircular zone compared with the control zone (Kruskal-Wallis, $F = 302.88$, df = 4, $P < 0.05$).

5.4.7. Effect on adult abundance. Field case study

From a total of 256 samples, 111 adult chrysopids belonging to four different species were captured (Table 5.4). *C. carnea* adults represented 91.9% of the total individuals and were the only species captured in June. Only a few *C. carnea* adults were collected on these first sampling dates (0.25 ± 1.05 captures per tree (mean ± SD) in control plots and 0.09 ± 0.42 in kaolin plots). However, the total number of *C. carnea* adults captured increased nearly nine-fold in October (GLMM, $Z = 6.62$, df = 252, $P < 0.01$), recording 0.86 ± 1.37 captures per tree in kaolin plots and 0.56 ± 1.10 in control plots. Overall, no significant effect of kaolin treatments on *C. carnea* adult abundance was detected (GLMM, $Z = -1.38$, df = 252, $P = 0.168$).

Table 5.4. Number of Chrysopidae adults captured by suction sampling in kaolin sprayed and water sprayed (control) olive trees in two different sampling events.

Species		June		October	
		Control	Kaolin	Control	Kaolin
Chrysoperla carnea (Stephens)	♀	5	1	19	24
	♂	3	2	17	31
Dichocrysa prasina (Burmeister)	♀	0	0	1	0
	♂	0	0	0	0
Dichocrysa flavifrons (Brauer)	♀	0	0	1	0
	♂	0	0	1	1
Chrysopa Formosa Brauer	♀	0	0	0	0
	♂	0	0	2	3
Total		8	3	41	59

5.5. Discussion

Although the kaolin-based particle film is considered to be an innocuous compound, its direct spray effect on insects varies depending on the test species. In psyllids, Hall et al. (2007) found no acute toxicity when spraying a 3% Surround WP suspension on Asian psyllid (*D. citri*) adults, nymphs and eggs. However, pear psylla nymphs and adults subjected to similar tests recorded significant mortality rates (Puterka et al., 2005). *Plutella xylostella* (L.) fourth instar mortality was just slightly increased by a particle film residual coating (Barker et al., 2006), and Larentzaki et al. (2008) reported that an interference with feeding capacity was the most probable cause of the higher mortality rates observed in kaolin-sprayed *T. tabaci*. In the case of *C. carnea*, direct kaolin applications do not appear to cause any direct mortality either in terms of interfering with feeding capacity or other effects that may reduce survival. Hall et al. (2007) suggested body size as a possible explanation for the differential effect on mortality among insect species. This opens up the possibility that early *C. carnea* first instars (around 2 mm),

which were not covered in this study, may be affected by residual kaolin coating. However, we have observed that ecdysis removes the kaolin coverage that remains attached to the molt. Therefore, we believe that kaolin particles have a zero, or insignificant, acute effect on *C .carnea* mortality. As for the ovicidal effect of kaolin, the results are consistent with experiments carried out on moth eggs unaffected by the kaolin cover (Barker et al., 2006; Unruh et al., 2000).

Although the kaolin residual coating did not affect mortality, the behavioral bioassay showed that kaolin-covered larvae were less mobile, slower, stopped more often, and thus travelled shorter distances. To the best of our knowledge, no such effects of kaolin residual coating have been previously reported for other insect species. Although the reason for these differences is not known, it might be related to the accumulation of kaolin particles in the articulations hindering normal walking activity. This fact could also help to explain the increase in turning activity detected. The difficulty of maintaining a straight walking line may be caused by the kaolin residue deposited on the legs. Due to the small differences between means, these results should be interpreted with caution. We cannot be sure about the actual consequences of the observed mobility reduction at a field scale. Chrysopid movement capacity is an essential element in their foraging efficiency, since prey encounters predominantly occur at random. It would be reasonable to suggest that kaolin particles adhering to the insect's surface may negatively influence prey searching and survival and therefore the overall biological control performance of the tested species. However, at field level, kaolin particle deposits will probably never reach the density assayed in the laboratory. Highly mobile chrysopid larvae may easily find shelter, thus partially avoiding the kaolin spray. By contrast, small reductions in mobility assessed at the laboratory scale over a reduced period of time may translate into a significant effect on the *C. carnea* larva life span.

Our grasping ability results are similar to those of the pear psyllid, which fell off treated surfaces as the kaolin particles tended to break away at the insect's grasping point (Puterka et al., 2005). However, falling off did not

occur in relation to psyllids on the underside of kaolin coated leaves, which is most likely due to the presence of conspicuous structures such as trichomes and stomates. Although attachment is expected to differ among insect species depending on their specific adaptations, *C. carnea* larvae seem to be affected in the same manner as psyllids. Under field conditions, grasping ability inhibition may result in an increase in the number of larvae falling from the tree canopy to the ground. *C. carnea* larvae are specifically adapted to minimizing the risk of dropping off plants. They produce an adhesive substance in their Malpighian tubules that is applied through the tip of the abdomen (1). The development of this adaptation is an indication that dislodgement from the plant represents a potential source of mortality (Rosenheim et al., 1999). The movement bioassay of larvae on a kaolin film revealed hampered locomotion on treated surfaces similar to that caused by direct spraying of larvae. These movement difficulties may induce the insect to preferably move on the control surface, thus explaining the differences in time spent in each zone. The presence of kaolin film has been reported to restrict the mobility of adult psyllids (Puterka et al., 2005) and neonate codling moth larvae (Unruh et al., 2000). *C. carnea* movement limitation on kaolin-covered surfaces, unlike restrictions due to insect coating film, can be regarded as a probable scenario in orchards. Moreover, the synergistic effects of both situations, which drastically hamper the insect's ability to forage, cannot be ruled out. By observing behavioral disturbances caused by kaolin films, Unruh et al. (2000) predicted that natural enemy disruptions are most likely to affect small insects that actively forage on the surface of treated plants. Our behavioral results bear out this assertion in the specific case of the predator *C. carnea,* where computerized video-tracking technology has proved to be a valuable methodological tool.

Insect oviposition deterrence has been acknowledged as one of main effects of particle films on pest insect behavior. (Lapointe et al., 2006; Larentzaki et al., 2008). Our findings indicate, on the contrary, that gravid *C. carnea* females manifest a preference for ovipositing on kaolin surfaces. This peculiar effect has previously been noted by Medina et al. (2007), who observed increased oviposition on kaolin coated twigs as compared to the water control. Kaolin particle deposits induce a structural modification in the

treated leaf surfaces that discourages certain insect ovipositions (Puterka et al., 2005). However, these modifications might elicit different behavioral responses depending on the egg species type. Consequently, *C. carnea* females may have chosen the most suitable anchoring substrate for their stalked eggs.

The literature available on the risks of kaolin treatment to beneficial arthropods is limited and inconclusive with respect to the concrete effect on chrysopid abundance. Marko et al. (2008) observed no difference between kaolin and control plots in terms of adult abundance, although the amount of lacewing larvae present was larger in kaolin-treated apple trees. Pascual et al. (2010) recorded a significantly smaller number of chrysopid captures on sprayed olive trees in one of the three years studied. However, as both larvae and adults were grouped together, the particular effects on the different life stages could not be identified. Our field experiment aimed to test the possibility that, given the results on oviposition preference, a kaolin film might exert an attractive influence on *C. carnea* adults. Even though adult chrysopids are present in olive orchards throughout the year (Campos, 2001), the small number of captured individuals on the first sampling date possibly coincided with a cyclic population decline in summer. In October, captures rose significantly, and, given that *C. carnea* males and females, contrary to previous reports, were numerically higher in kaolin plots, single kaolin treatments do not appear to affect *C. carnea* adult presence in the short term. Our results regarding adult oviposition and abundance reveal that further research is required at field level in order to clarify adult lacewing responses to this compound.

Acknowledgements

The authors would like to thank María Luisa Fernández Sierra, Damián Martínez Valdivieso and Herminia Barroso for their laboratory assistance and Dr. Víctor Monserrat for his guidance on lacewing identification. We would also like to thank Daniel Paredes, Juan Castro and the Public Company for Agricultural and Fisheries Development of Andalusia for their contributions to the field experiment carried out in the experimental orchard provided by the Junta de Andalucía. This study was grant-aided by project P07-AGR-02747

(Junta de Andalucía) and project AGL 2005-00932 (Ministry of Science and Education). Finally, we would like to thank Michael O'Shea for proof-reading the document.

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6. Agricultural management systems affect the green lacewings community (Neuroptera: Chrysopidae) in southern Spanish olive orchards

Agricultural management systems affect the green lacewings community (Neuroptera: Chrysopidae) in southern Spanish olive orchards

Under revision in *Environmental Entomology*

6.1 Abstract

This study aims to establish the effects of (1) herbicidal weed cover removal and insecticide applications and (2) the general farm management systems used in olive orchards of southern Spain on chrysopid assemblages and abundance. Over two consecutive years (1999 and 2000), green lacewing adults and larvae were collected from three olive orchards under conventional, integrated and organic management systems. On each sampling occasion, soil and twig samples were taken with the objective of determining insecticidal and herbicidal treatments through chemical analysis of residues. In year 2003, chrysopids were collected in May and June from 9 orchards (three per management system). Eight adult species and three genera of larvae were identified. No rare species were captured from the most intensively farmed orchard, which thus recorded the most limited chrysopid diversity with a very marked dominance of *Chrysoperla carnea s.l.* No effect of dimethoate treatments on *Chrysoperla* larvae and *C. carnea* adults was observed. However, the presence of insecticide residues was related to *Dichochrysa* larvae depletion. The absence of herbicide treatments favoured *C. carnea s.l.* adults on olive trees while larval abundance decreased. *Dichochrysa* larvae were more abundant when weed cover received no treatment. In 2003, there was no difference in *Chrysoperla* spp. larvae abundance between conventional and organic orchards. However, in orchards under organic management, *Dichochrysa* larvae were more abundant. The implications for biological control and conservation of chrysopid species are discussed.

Key words: *Chrysoperla carnea*, *Dichochrysa,* generalized additive model (GAM), herbicide, pesticide

6.2 Introduction

Lacewing larvae of the Chrysopidae family are voracious polyphagous feeders that exert biological control as predators of a wide range of pest arthropods in crops (Stelzl and Devetak 1999, Duelli 2001). In olive orchards, chrysopids represent a diverse complex of species, are present during almost the entire year and consume a number of soft-bodied pest arthropods such as the black olive scale, the olive psylla and the olive moth (Szentkiralyi 2001). Chrysopids' major contribution to olive crop protection is their predatory activity on the eggs of olive moths (*Prays oleae* Bern.) (Ramos et al. 1984, Campos 2001) by preventing these eggs from hatching which may eventually trigger fruit falls. The potential of green lacewings for pest suppression and their natural occurrence in olive orchards make them ideal candidates for conservation biological control in IPM programs (Corrales and Campos 2004). The conservation approach seeks to enhance the efficiency of natural enemies by modifying their habitat and rectifying negative pesticide practices (Barbosa 1998, Jonsson et al. 2008). In this regard, organic management with no chemical insecticide use and greater habitat heterogeneity has been observed to promote natural enemy abundance and evenness (Schmidt et al. 2005, Crowder et al. 2010). However, ecologically sound agricultural practices are not confined to organic farming systems, and it is therefore important to establish which aspects of management determine the response of natural enemies and to what extent they do so (Garratt et al. 2011). Although Corrales and Campos (2004) investigated the response of *C. carnea s.l.* adults to different management systems used in olive orchards, little is known about their impact on other chrysopid species and larval abundance.

Preliminary observations have indicated that different olive cropping management systems might influence green lacewing species assemblages (Ruano et al. 2001). Hence, this study aims to (1) establish the effect of insecticide usage and the herbicidal removal of weed cover on chrysopid abundance and diversity in olive orchards and (2) to assess the response of chrysopids to different management systems in relation to the agricultural practices considered.

6.3 Materials and methods

6.3.1 Study sites

Chrysopids were collected during years 1999, 2000 and 2003 in the province of Granada (as defined by Spanish government boundaries), one of the largest commercial olive-producing areas in southern Spain. This region is characterized by a very high proportion of olive orchards with a similar structure, vegetable crops and a limited number of patches of non-agricultural land dominated by indigenous shrubland vegetation. The size and number of patches of Mediterranean oak forest land in the region are limited. In 1999 and 2000, three orchards located approximately 4 km apart, with identical climatic conditions and at roughly similar altitudes of between 700 and 1000 masl., were selected according to agricultural intensification criteria. All three orchards were planted in plots 10 × 10 m apart with cv. Picual olive trees of similar size and age (between 50 and 80 years old). The first site, under conventional management (hereafter referred to as CV), was systematically treated with insecticide throughout the year against the olive moth, *Prays oleae* (Bernard), and the olive fruit fly, *Bactrocera oleae* (Gmel.). Simazine herbicide treatments were applied twice a year for weed control. The second site (IT) had been under an IPM system for the previous 15 years. Pest control relied upon technical recommendations based on specific thresholds and, according to the farmer, a single dimethoate treatment was carried out during the whole sampling period in June 2000 against *P. oleae*. Weeds were controlled using a simazine soil spray in March and optionally in September. The third site was a certified organic orchard (OR) that had been under this type of management for the previous 8 years. No pest control strategies were used, and the naturally occurring cover was removed by shallow plowing from late May to the beginning of June. In 2003, three orchards per management type (conventional, integrated and organic), including the orchards sampled in 1999 and 2000, were studied in order to determine whether the general management system used affected the abundance and diversity of chrysopids. The additional orchards studied presented management characteristics similar to those described above. Conventionally managed orchards received systematic pesticide and herbicide treatments. The organic orchards received no chemical treatments, and all three had natural weed cover. The integrated

orchards were treated only under certain infestation conditions and just one of the orchards had managed weed cover.

6.3.2 Collection of Chrysopidae

In 1999 and 2000, samples were taken monthly between March and October. In 2003, the orchards were sampled twice, in mid-May and mid-June, coinciding with the most important predatory activity of chrysopids on *P. oleae* eggs laid on the olive fruit (Campos 2001) and the largest difference in arthropod abundance between management systems in olive orchards (Ruano et al. 2004). Olive trees were sampled using the beating technique. Branches were shaken five times over a 50 cm sweeping net and the dislodged arthropods were collected. One branch was randomly sampled per cardinal direction, with up to a total of four branches being sampled per tree. A block consisted of 5 sampled trees 20 m apart situated in the same row (one tree was left unsampled among the sampled trees). Six blocks were sampled per site and month in 1999, which was reduced to five blocks in 2000 and four in 2003 following a recalculation of the sample size (Ruano et al. 2004). Blocks were located at a minimum of 500 m apart. The samples were frozen, and the chrysopids, larvae and adults were subsequently separated from the vegetal and inorganic remains. Adults were identified to species level and larvae to genus level under a stereomicroscope (Stemi SV8; Zeiss, Oberkochen, Germany) with the help of training provided by a chrysopid taxonomy specialist (V. Monserrat) and keys provided by Díaz-Aranda et al. (2001). Our problematic and rare species identifications were later confirmed by this specialist.

6.3.3 Determination of agrochemicals

In 1999 and 2000, on each sampling occasion, olive twigs were randomly collected from six olive trees in each site. Twigs from the same site were pooled and frozen at −18° C for conservation until their extraction. Ten soil samples were collected along straight transects 10 m apart. Samples from

the same site were pooled and the field-moist soil was stored at 4 ºC until analysis.

Simazine was extracted from soil samples (ca. 20 g) using Soxhlet for 2 h with 75 mL ethyl acetate. The extract was concentrated to 1-2 mL in a rotary evaporator at 40 ºC, concentrated to dryness under a gentle stream of nitrogen and redissolved in 1 mL hexane. Dimethoate was extracted from olive twigs as described in Peña et al. (2006). Pesticide analysis was carried out by gas chromatography with a thermionic specific detector (from Varian) on a HP-5 column. Calibration of the response, assayed with pesticide standards, resulted in $R^2 > 0.99$ in all cases. Recovery of simazine from spiked soil ($n = 4$) was 97.7%.

Chemical residues confirmed the application of dimethoate insecticide and simazine herbicide in sites CV and IT during years 1999 and 2000. CV presented dimethoate residues in May and June 1999 due to a single application carried out in that year. In 2000, however, pesticide residue was present every month from May to October and two applications were detected during this period in June and October. In site IT, a single pesticide treatment in July 2000 was recorded during the whole sampling period. In relation to weed control, two herbicide treatments were systematically carried out in 1999 and 2000 in site CV in March and June, and residues in this site were therefore detected on every sampling occasion with the exception of October 1999. Similarly, in site IT, weeds were treated twice in 1999 (March and October) and residues were detected throughout the sampling period. Unlike in 1999, weed control in 2000 was carried out only in early spring, with a single application detected in March. Therefore, from the month of June until the end of the sampling season, site IT remained residue free. No agrochemical residues were found in site OG during the sampling period. Full details of insecticide and pesticide residues detected in the orchards sampled in 1999 and 2000 have been presented by Ruano et al. (2004).

6.3.4 Statistical analysis

Generalized additive models (GAMs), with estimations based on a penalized regression spline approach, were used to evaluate the influence of pesticide and herbicide presence on chrysopid abundance in the data for 1999 and 2000. GAMs were chosen due to their usefulness in modeling trends in time-series data (Benton et al. 2002). These models assume that the mean of the response variable depends on an additive predictor through a nonlinear link (Hastie and Tibshirani 1990). This extends generalized linear models (GLMs) by allowing for non-linear relationships between the response variable and the explanatory variables as in the case of the relationship between time and insect abundance. In order to construct the models, adult and larval abundance data for each individual block on a site in each sampling month were taken as an experimental unit. Before analysis, the data were checked for temporal autocorrelation using autocorrelation function (ACF) plots for regularly spaced time series (Zuur et al. 2010). Different models were fit to the abundances of (a) *C. carnea s.l.* adults, (b) *Chrysoperla* larvae and (c) *Dichochrysa* larvae. The *C. carnea s.l.* adult model included the explanatory variables: (1) insecticide presence-absence, (2) herbicide presence-absence, (3) site, (4) month, (5) year, and significant second-order interactions between parameters. In addition to these variables, the two models for larvae included: (6) same genus adult abundance and (7) the abundance of other genera of larvae as explanatory variables for testing the influence of adult presence on sampled larval abundance and possible interspecific competition effects. The response to the month variable was modeled as a smooth function using cross-validation to automatically estimate the amount of smoothing (Wood 2006). The three models assume a similar pattern of seasonal abundance in both 1999 and 2000.

Models were selected by combining forward and backward selection based on Akaike Information Criteria (AIC) (Zuur et al. 2007). Both *Chrysoperla* (adults and larvae) models were analyzed using negative binomial error distribution due to over-dispersion. The *Dichochrysa* larvae model was constructed using a Poisson distribution and was corrected by applying a quasipoisson model. Adult individuals of species belonging to the *Rexa*,

Chrysopa and *Dichochrysa* genera and larvae of the *Rexa* genus were not sufficiently abundant to permit an adequate model to be constructed and therefore were not tested (Table 6.3).

In addition, the Principal Component Analysis (PCA) ordination technique was applied to adult species and larval genera captures in 1999 and 2000 to graphically represent multivariate patterns in chrysopid assemblages across sites and years and the association of sites and species. Before the analysis was carried out, the data was checked for linear response (Lepš and Šmilauer 2003), centered, and $\sqrt{x}$ transformed to down-weight the effect of abundant species. PCA was based on the covariance matrix (scaling 1). The diversity of chrysopid adult species over the entire sampling period was calculated using Shannon's index and the Dominace index using EcoSim 7.71. Adult individuals sampled during 1999 and 2000 were pooled as only three species were observed in 1999 possibly due to the small overall number of adult captures compared to 2000 (Table 6.1).

The abundance of *Chrysoperla* and *Dichochrysa* larvae captured in 2003 was analyzed using a generalized linear mixed model (GLMM) with a Poisson error distribution corrected using a quasipoisson model (R software 2.1.2, package MASS). Management system (conventional, integrated and organic) and month were used as explanatory variables. Site was established as a random effect due to the large number of different orchards sampled. Adult individuals captured in 2003 were insufficient to produce an appropriate model and therefore were not tested (Table 6.1). A multivariate PCA based on the covariance matrix was performed using adult species and larval genera captured in 2003 to assess dissimilarity in assemblages as a result of the different management systems. Data was checked and transformed as described above and one-way multivariate analysis of variance (MANOVA) was then carried out, with management system as a fixed factor and first and second axis PCA scores as dependent variables, in order to determine if management system separations were statistically significant. Tukey's HSD post hoc test was used for pairwise comparison of management systems. Before the analysis, the data was tested for MANOVA assumptions using the

energy test of multivariate normality (R software 2.1.2, Energy package) and Box's test of equality of covariance matrices (SPSS Statistics 18 for Windows).

6.4 Results

6.4.1 Chrysopid diversity

A total of 758 green lacewing larvae and 531 adults were collected during the sampling periods of 1999, 2000 and 2003.

Years 1999-2000. Larvae from 3 genera and 8 adult chrysopid species were captured between March and October (Table 6.1). The larvae genera identified were *Chrysoperla*, *Dichochrysa* and *Rexa*. *Chrysoperla* was the most abundant genus in all three sites. It was the only genus present in site CV, the most abundant in site IT (81.3%) and accounted for more than half of the total chrysopid larvae captured in site OG (56.6%). *Dichochrysa* was considerably more abundant in OG (43.4%) than in IT (12.1%) and was not captured in CV. *Rexa* was exclusively present in IT but in small numbers (6.0%).

Three species (*C. carnea s.l.*, *D. prasina* and *D. genei*) of chrysopid adults totaling 144 individuals were observed in 1999, with *C. carnea s.l.* being the most abundant species (Table 6.1). However, the largest number of species was observed in 2000, when 8 different green lacewing species with a total of 392 individuals were identified. Overall, *C. carnea s.l.* was the most abundant species, followed by *D. prasina*, while very small numbers of the other species were captured (Table 6.1). *Rexa lordina* adults, as in the case of larvae, and *D. genei* were only present in site IT (Fig. 6.1). Species *C. viridiana*, *D. flavifrons* and *D. picteti* were captured exclusively in site OG. Thus, rare or casual species, according to the classification given in Canard et al. (2007), were only found in sites IT and OG (Fig. 6.1).

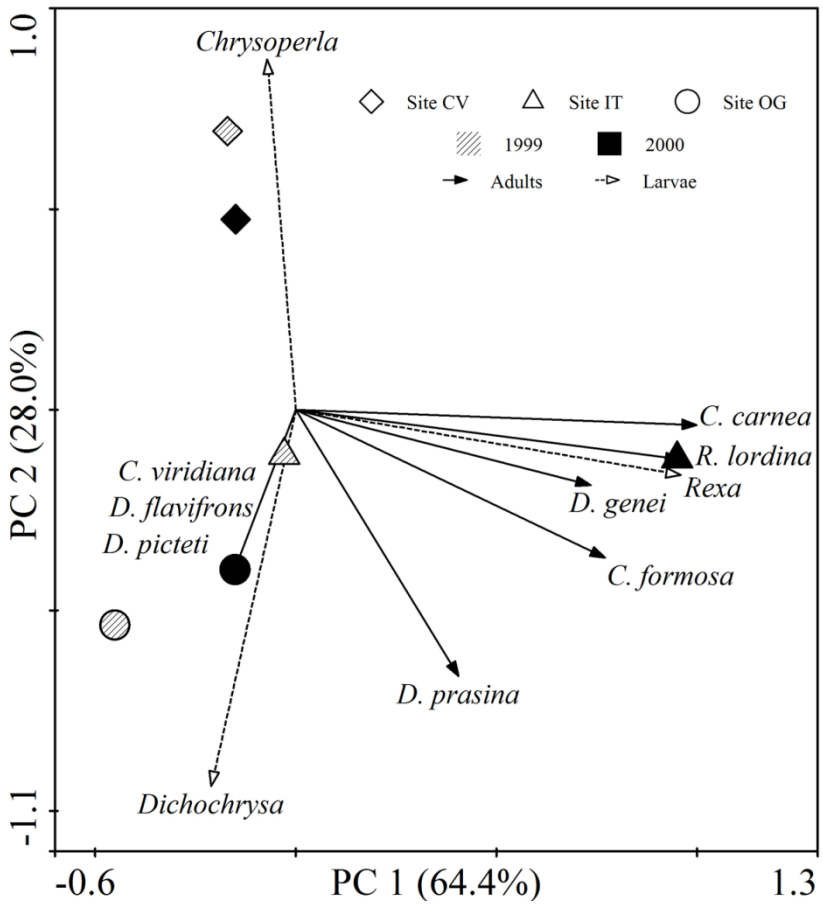

Figure 6.1. PCA biplot of green lacewing adult species and larvae genera loading and site scores in years 1999 and 2000.

Table 6.2. Biodiversity indices calculated during the whole 1999 - 2000 sampling period for each site.

Index	CV	IT	OR
Species richness	2	5	6
Shannon	0.071	0.183	0.648
Dominance	0.986	0.967	0.846

(CV) Conventional orchard (IT) integrated orchard and (OR) organic orchard.

Site OG was the olive orchard with the highest diversity value, the richest in chrysopid species and the lowest dominance of *C. carnea s.l.* (Table 6.2). On the other hand, only one individual not belonging to species *C. carnea s.l.* was collected in site CV during the two-year period, which was therefore the least diverse and showed extreme *C. carnea s.l.* dominance. Several

species were captured in site IT, although *C. carnea s.l.* was very abundant, which increased its dominance and consequently reduced the site's biodiversity.

Year 2003. In May and June 2003, total captures of individual larvae from two genera (*Chrysoperla* and *Dichochrysa)* reached 153 (Table 6.1). Only two species of adults were identified with a total of 27 green lacewings being captured.

6.4.2 Temporal distribution

*Years 1999-2000***.** The collection of adults and larvae began to be effective from the month of May. As modeled by the GAMs, seasonal abundance of *C. carnea s.l.* adults and *Chrysoperla* and *Dichochrysa* larvae recorded significant changes from May to October (Table 6.1). The adult abundance of *C. carnea s.l.* showed an upward trend throughout the sampling period and peaked in June and October (Fig. 6.2 D). Higher capture rates were recorded in 2000 (Table 6.1), especially in the month of September (Fig.6.2 A). *Chrysoperla* larvae recorded notable abundance maxima in June and at the end of August (Fig. 6.2 E). In addition, although adult abundance levels differed in 1999 and 2000, no interannual variation was observed in the case of larvae (Fig. 6.2 B). *Dichochrysa* larvae were significantly more abundant in 1999 (Fig. 6.2 C) and increased from May onwards, reaching a single peak in August (Fig. 6.2 F). *Dichochrysa* larvae presence showed a significant positive correlation with *Dichochrysa* adult abundance (estimate = 0.692, Table 6.1). Although this correlation was also observed in the case of *Chrysoperla* larvae and *C. carnea s.l.* adults, the relationship was much weaker. Neither of the two larvae models showed a statistical relationship between *Dichochrysa* and *Chrysoperla* larvae presence.

Table 6.1. Abundance and proportion of each green lacewing adult species and larvae genera captured in the different sampling periods.

Taxonomic status[a]	1999 (Mar. to Nov.)		2000 (Mar. to Nov.)		2003 (May to Jun.)	
	Abund.	%	Abund.	%	Abund.	%
Adults						
Gen. *Chrysoperla* Steinmann, 1964						
C. carnea s. l. (Stephens, 1836)	105	94.6	373	95.6	25	92.6
Gen. *Dichochrysa* Yang, 1991						
D. prasina (Burmeinster, 1839)	5	4.5	7	1.8	2	7.4
D. flavifrons (Brauer, 1850)	0	0	3	0.8	0	0
D. picteti (McLachlan, 1880)	0	0	1	0.3	0	0
D. genei (Rambur, 1842)	1	0.9	1	0.3	0	0
Gen. *Chrysopa* Leach in Brewster, 1815						
C. formosa Brauer, 1850	0	0	3	0.8	0	0
C. viridiana Schneider, 1845	0	0	1	0.3	0	0
Gen. *Rexa* Navás, 1919						
R. lordina Navás, 1919	0	1.0	1	0.3	0	0
Larvae						
Gen. *Chrysoperla* Steinmann, 1964	275	79.5	193	84.6	98	64.1
Gen. *Dichochrysa* Yang, 1991	70	20.2	26	11.4	55	35.9
Gen. *Rexa* Navas, 1919	1	0.3	9	3.9	0	0

[a] All species belong to the subfamily *Chrysopinae* Esben-Petersen, 1918, Trib *Chrysopini* Schneider, 1851.

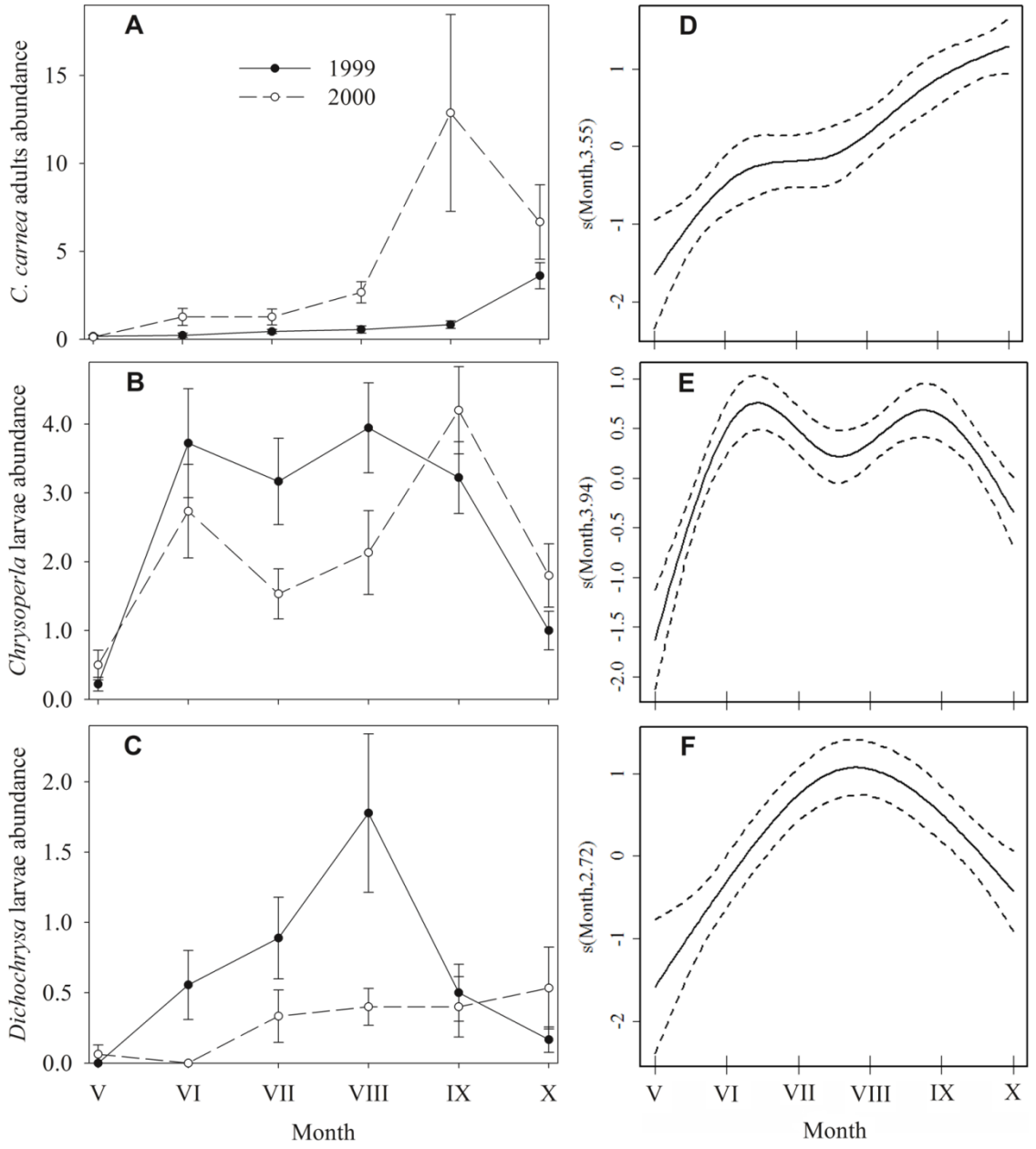

Figure 6.2. Temporal distribution of *C. carnea s.l.* adults (A) and *Chrysoperla* and *Dichochrysa* larvae (B and C) captures per block in 1999 and 2000, and smoothers with 95% pointwise confidence bands from *C. carnea* adult (D) and *Chrysoperla* and *Dichochrysa* larvae (E and F) GAMs, representing the modeled impact of temporal distribution on captures. The estimated degrees of freedom are shown in the y-axis label. Smoother *P*-values and effect of year *P*-values are shown in Table 6.1.

Adult individuals from the *Dichochrysa* genus showed no clear distribution pattern, possibly due to the low capture rate. *Chrysopa* spp. adults were exclusively present in June and July, while *Rexa lordina* larvae and adults appeared in both years only during the months of May and June.

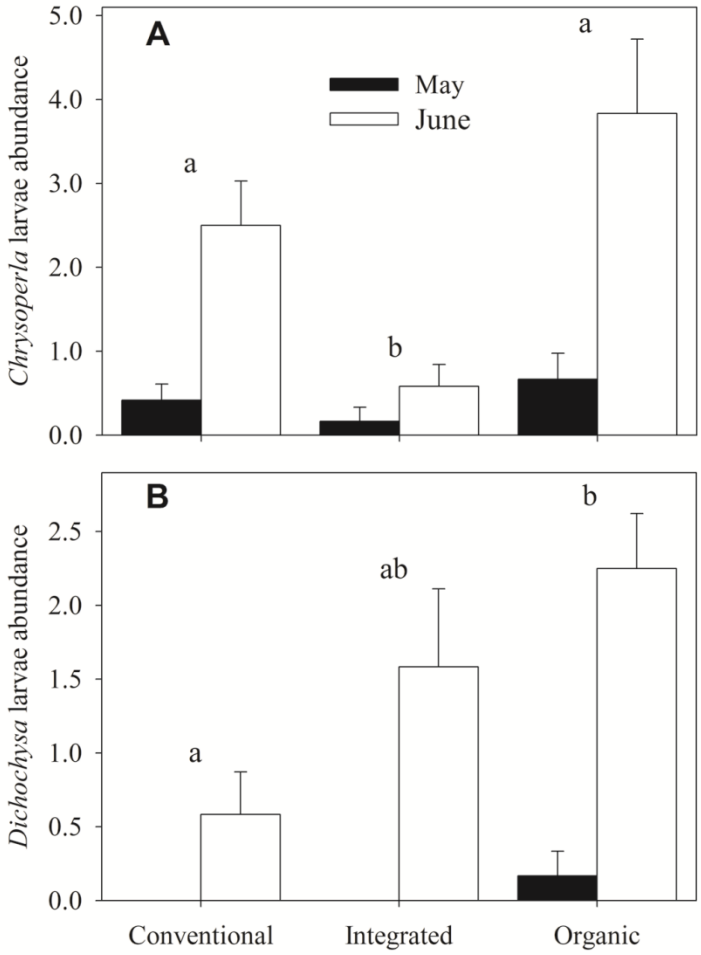

Figure 6.3. Abundance per block recorded in 2003 of *Chrysoperla* larvae (A) and *Dichochrysa* larvae (B) present in orchards under different management systems. Bars with different symbols indicate statistically significant differences ($P < 0.05$).

Year 2003. Individuals from the two genera of larvae identified (*Chrysoperla* and *Dichochrysa*) were more abundant in mid-June than in mid-May (Fig. 6.3) (GLMM, $t = -4.96$, df = 62, $P < 0.001$ and $t = -3.85$, df = 62, $P < 0.001$, respectively). This finding is in line with the results for seasonal abundance trends obtained in 1999 and 2000 (Fig. 6.2 E and F).

6.4.3 Insecticide application and herbicidal weed removal

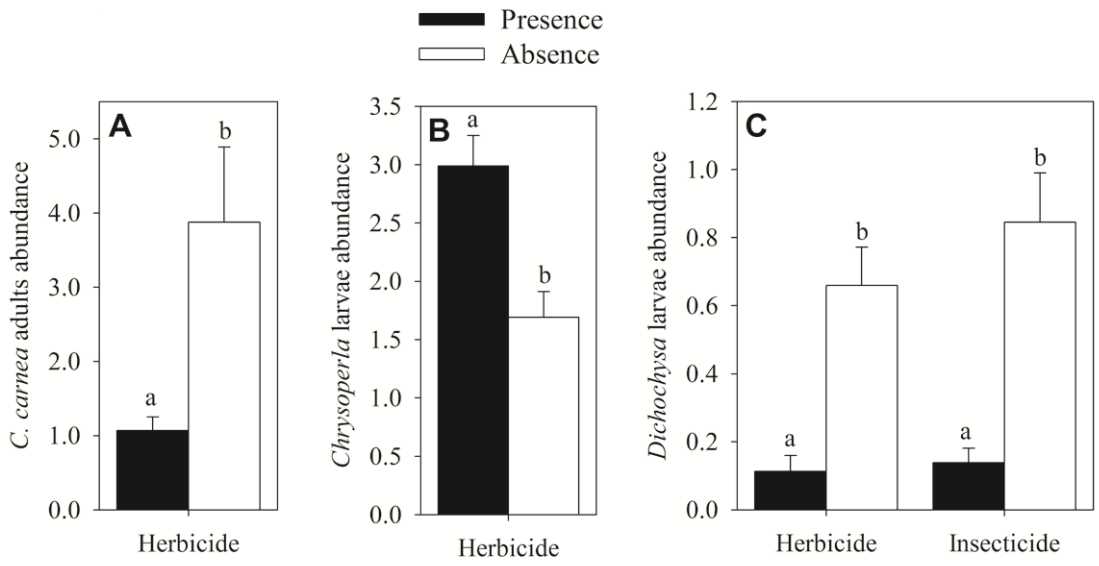

Figure 6.4. Abundance per block recorded in 1999 and 2000 of *C. carnea* adults (A), *Chrysoperla* larvae (B) and *Dichochrysa* larvae (C) in relation to the presence or absence of herbicide and/or insecticide found to be significant by the GAMs (Table 6.1). Bars with different symbols indicate statistically significant differences ($P < 0.05$).

No effect of insecticide treatments on *C. carnea* adult abundance was observed in years 1999 and 2000 as the variable was not significant and was therefore excluded from the final model (Table 6.3). However, simazine herbicidal treatments negatively affected the presence of common green lacewing adults (Fig. 6.4 A), with a considerably higher capture rate being recorded when herbicide residues were not present. In this regard, it is important to mention that *C. carnea* adults were massively captured in site IT during September (35.80 ± 25.49 captures per block, mean ± SD) and October (17.00 ± 5.34 captures per block) in 2000, coinciding with a period when, as described previously, no weed control treatment was carried out in this site. IT was also observed to have a higher abundance of *C. carnea* adults as compared to sites CV and OG, which cannot be explained by the other variables used in the model (Table 6.3). *Chrysoperla* larvae abundance, as in the case of *C. carnea* adults, did not respond to the presence of insecticide residue. In herbicide-treated orchards, larvae, unlike adults, were more abundant (Fig. 6.4 B). *Chrysoperla* larvae showed a higher rate of natural abundance in site CV as compared to the other two sites. No significant differences were observed between sites IT and OG. Herbicide and insecticide treatments significantly influenced the presence of *Dichochrysa* larvae in the

three orchards sampled during 1999 and 2000 (Table 6.3 and Fig. 6.4 C). *Dichochrysa* larvae abundance was lower in sites and months with a detectable presence of insecticide and herbicide. As the GAM identified these factors as the main explanatory variables, the site variable, despite the differences in abundance, was not significant in the model.

Table 3. GAM results for *C. carnea* adults and *Chrysoperla* and *Dichochrysa* larvae.

		Estimate	SE	x^2	df	*P*-value
Adults						
	C. carnea					
	Herbicide (P)	– 0.893	0.279	– 3.199	1	0.001
	Site (IT) [a]	0.693	0.255	2.722	1	0.006
	Site (OG) [a]	– 1.001	0.344	– 2.924	1	0.003
	year (2000)	1.000	0.213	4.700	1	0.005
	s (month)			86.810	3.55	< 0.001
Larvae						
	Chrysoperla					
	Herbicide (P)	0.561	0.238	2.356	1	0.018
	C. carnea adults	0.032	0.008	3.842	1	< 0.001
	Site (IT) [b]	– 0.489	0.171	– 2.831	1	0.005
	Site (OG) [b]	– 0.350	0.279	– 1.254	1	0.210
	s (month)			63.500	3.94	< 0.001
	Dichochrysa					
	Insecticide (P)	– 1.012	0.440	– 2.300	1	0.021
	Herbicide (P)	– 2.015	0.297	– 6.773	1	< 0.001
	Dichochrysa adults	0.692	0.327	2.120	1	0.034
	year (2000)	– 0.972	0.265	– 3.669	1	< 0.001
	s (month)			32.930	2.72	< 0.001

No significant interactions between variables were obtained for any of the models.

(P) indicates presence.

[a] Pairwise comparisons of sites for *C. carnea* GAM: Site IT - Site OG, Estimate = -1.698, SE = 0.265, x^2 = – 6.41, *P* < 0.001.

[b] Pairwise comparisons of sites for *Chrysoperla* GAM: Site IT - Site OG, Estimate = -0.139, SE = 0.246, x^2 = 0.56, *P* = 0.572.

6.4.4 Management system

In 2003, *Chrysoperla* larvae showed the highest level of abundance in June and May in organically managed orchards (Fig. 6.3 A). However, no difference was observed between organic and conventional farms (GLMM, t = 1.10, df = 6, P = 0.31). The number of captures registered in integrated orchards was significantly smaller than those registered in conventional (GLMM, t = - 2.56, df = 6, P = 0.043) and organic management systems (GLMM, t = -3.43, df = 6, P = 0.014). As with *Chrysoperla*, the abundance of *Dichochrysa* larvae showed the highest levels in organic orchards and differed greatly from those registered in conventionally managed farms (GLMM, t = 2.86, df = 6, P = 0.029). *Dichochrysa* larvae abundance in orchards under the IPM system was not significantly different from levels in conventionally managed (GLMM, t = - 1.91, df = 6, P = 0.105) and organic orchards (GLMM, t = 1.21, df = 6, P = 0.271) (Fig. 6.3 B).

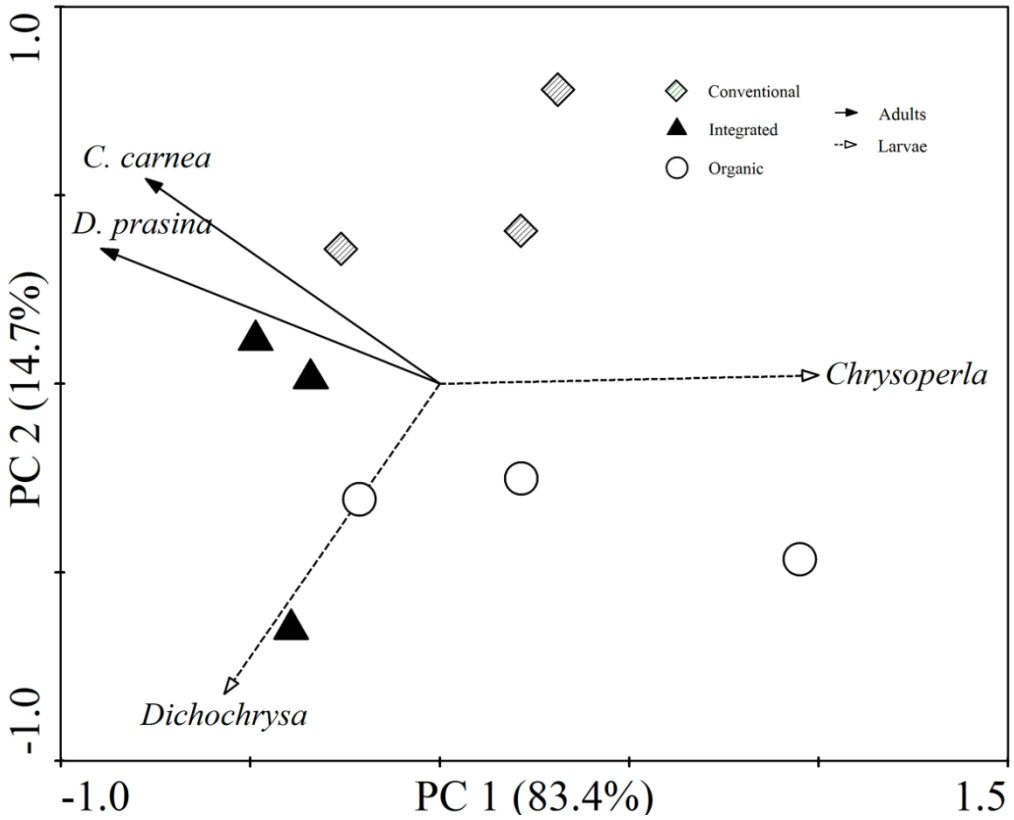

Figure 6.5. PCA biplot of green lacewing adult species and larvae genera loading and site scores in the year 2003.

The PCA ordination biplot, that included larvae genera and adult species captured in the nine sites during 2003, accounted for 98.1 % of the total variance (Fig. 6.5). The plot clearly differentiated between assemblages from conventionally managed orchards and the other management systems, and overall MANOVA showed statistically significant differences in PC scores (MANOVA, $F = 4.51$, df = 4,12, $P = 0.019$). These differences were specifically observed along PC 2 (ANOVA, $F = 7.75$, df = 2, $P = 0.022$), where conventional orchard scores differed significantly from those for organic orchards (Tukey's HSD, $P < 0.05$). These differences observed along PC 2 are mainly due to the high loading rates of *Dichochrysa* larvae (Figure 6.5). No significant differences were observed along PC 2 between integrated orchards and the other management systems (Tukey's HSD, $P > 0.05$). Orchard scores along PC 1 with a high loading rate of *Chrysoperla* larvae did not differ (ANOVA, $F = 2.77$, df = 2, $P = 0.141$).

6.5 Discussion

Chrysopids as a group have generally been regarded as relatively resistant to insecticide applications (Pappas et al. 2011). In olive trees at field level, Santos et al. (2007) observed that neither adult nor larvae abundance was significantly affected by a single dimethoate application and reported that this finding might be related to the known resistance of *C. carnea* to insecticidal compounds.

Various studies have highlighted the ability of *C. carnea* to show a detoxifying response to insecticides as adults (Mulligan et al. 2010) and larvae (Ishaaya and Casida 1981) as a result of increased levels of non-specific esterases. Our results regarding *C. carnea* abundance in relation to insecticide presence confirm that insecticide applications have little effect on the larval and adult stages of this species. However, our findings suggest that species of the Chrysopidae family may differ significantly in their response to insecticides. *Dichochrysa* larvae suffered a severe population reduction due to dimethoate treatments. Furthermore, a single adult of *Dichochrysa prasina* was captured in the heavily treated CV site, while greater abundance and diversity of *Dichochrysa* spp. adults were recorded in the other sites. Most of the extensive laboratory research on pesticide side effects has actually focused

on *C. carnea* (Vogt et al. 2001) and, more recently, on other species of the *Chrysoperla* genus (Chen and Liu 2002, Bueno and Freitas 2004). However, to date, there is no information available on the compatibility of pesticide use with other chrysopid species of economic importance such as *D. prasina,* and, according to our findings, the results of toxicity studies carried out on *C. carnea* cannot be extrapolated to closely related genera.

Dichochrysa larval populations were also affected by weed cover treatments applied to the soil. Unfortunately, although no models for the different species of adults could be constructed, the positive relationship with *Dichochrysa* larvae observed suggests that one or more species of the genus may be influenced by the presence of natural weed cover. In addition, a very small number of *Dichochrysa* spp. adults was captured as compared to the number of larvae, indicating that most of the adults were not present in the olive trees at the time of sampling and may have preferred nearby vegetal substrates instead. Weed cover presence was found to positively influence the presence of *C. carnea* adults which were considerably more abundant when cover was not removed. The presence of pollen and nectar-rich flowers that provide the nutrients required by the common green lacewing (Villenave et al. 2006) may have exerted an attraction effect which contributes to the establishment of adults in the area. On the other hand, larvae abundance did not respond in the same way despite the positive relationship observed between *Chrysoperla* larvae and *C. carnea* adults. Although the reason for this is unknown, as discussed by Jacometti et al. (2010) in relation to brown lacewings, it may be due to an indirect effect of weed cover that possibly contributes to increasing the abundance of chrysopid natural enemies such as parasitoids, known to be population-limiting factors for lacewings in olive orchards (Campos 2001). In addition, in 2003, the largest amount of *Chrysoperla* larvae was collected from organically managed olive orchards, all of which had weed covers. This is inconsistent with the results obtained for the 1999-2000 period, thus backing up the hypothesis that the negative effect may be mediated by an indirect factor that is not always present.

The results obtained in 2003 for *Dichochrysa* larvae under different management systems are highly consistent with their susceptibility to dimethoate applications and the positive effect of managed weed cover

detected in years 1999 and 2000. The differences observed between management systems are therefore clearly related to the combination of insecticide use and lack of weed cover. No relationship between management and biodiversity could be established in 2003 since the sampling period lasted for only two months and the number of adults captured was very low. However, rare species captures and biodiversity indices for the three orchards sampled in 1999-2000 suggest a negative correlation with agricultural intensification which could not be tested statistically due to the limited number of individuals observed. The limited presence of *Dichochrysa* larvae in conventional orchards during the three years of the study may also support this hypothesis, as *Dichochrysa* spp. account for 13 out of the total of 31 species of chrysopids studied in olive orchards worldwide (Szentkiralyi 2001). This reduction in chrysopid diversity has also been documented by Corrales and Campos (2004) who sampled *C. carnea* adults from the same sites by using McPhail traps and observed very high dominance levels for *C. carnea* in site CV as compared to sites IT and OG. Our results show that the agricultural practices considered in this study depleted the presence of green lacewing larvae of the *Dichochrysa* genus, which are potentially important in terms of numbers and species diversity in olive orchards. This reduction in chrysopid predators may translate into decreased biological control in orchards under intensive management, as has been observed in the past for predatory activity against *P. oleae* (Ramos et al. 1978). However, as chrysopids are functionally redundant, the depletion of some species in the chrysopid community may lead to density compensation by *Chrysoperla* spp., and therefore pest suppression capacity may not be affected (Straub et al. 2008). Resistant *C. carnea* populations have been observed to possess great predatory potential (Pathan et al. 2010). Nevertheless, intensive agricultural practices in olive orchards clearly appear to have negative implications for rare chrysopid species conservation, as in the case of the *R. lordina* species, which is particularly associated with olive crops (Monserrat 2008).

Acknowledgements

We wish to thank Dr. Víctor Monserrat for his contribution to lacewing identification and Herminia Barroso and Maria Luisa Fernández Sierra for their assistance. We would also like to thank Michael O'Shea for proof-reading

the manuscript. The study was supported by the Spanish Ministry of Education (projects REN2002-03269 and AGL2005-00932) and the Junta de Andalucía (project P07-AGR-02747).

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7. A managed resident vegetation cover contributes to increase the abundance of green lacewings (Neuroptera: Chrysopidae) on olives trees

A managed resident vegetation cover contributes to increase the abundance of green lacewings (Neuroptera: Chrysopidae) on olives trees

In preparation for *Biological Control*

7.1 Abstract

Understorey habitats are optimal ecological structures for natural enemy enhancement in fruit orchards. A large scale experiment was carried out to establish the effect of resident vegetation cover (VC) on green lacewings as compared to maintaining bare soil (BS), the dominant soil management strategy used in Spanish olive orchards. Lacewings were sampled using baited McPhail traps for adults, and suction was used to collect adults and larvae from olive canopies. Additionally, we monitored the presence of the lacewing's principal target pest, olive moth eggs, as well as VC composition and density. McPhail trapping showed significantly higher chrysopid abundance levels in VC plots during the two years covered by the study even though flowering plants represented 29.7% of total plant species. Multivariate analysis identified *Chrysoperla carnea s. l.* and *Dichochrysa prasina* as contributing to differences in abundance. VC slightly increased capture diversity. However, no specific link between any of the species and VC was detected. No differences were observed in individuals collected through suction in 2009, which could be attributed to low sampling efficiency. In 2010, when sampling was increased considerably, higher adult and larvae abundance levels were recorded in VC only with respect to *C. carnea s.l.* A delay was detected between McPhail captures and suction collection peaks. The fact that VC promoted higher abundance detected earlier through trapping, and later on olive canopies through suction, coinciding with *P. oleae* presence, suggests that resident VC may contribute to a build-up of green lacewing populations moving onto the crop at the time of the pest attack.

Key words: *Chrysoperla carnea*, *Dichochrysa prasina,* habitat management generalized additive mixed model (GAMM), predator, weed cover

7.2 Introduction

Habitat management of agricultural ecosystems is a subset of conservation biological control methods aimed at increasing the abundance and fitness of native natural enemies by delivering resources at the optimal temporal and spatial scales (Landis et al., 2000; New, 2005). Of the different options available, within-field habitats have been demonstrated to play an important role in predator and parasitoid enhancement on agricultural sites (Bianchi et al., 2006; New, 2005; Tscharntke et al., 2005). Non-crop vegetation provides refuge, alimentary resources and alternative prey and also facilitates contact between natural enemies and their target pests. Natural enemies can, in turn, increase pressure on insect pest populations, thus reducing the pesticide input required for their control (Thomson and Hoffmann, 2009). Arboreal crops such as fruits are characterized by a multi-strata structure comprising an understorey habitat and tree canopies (Simon et al., 2010). The understorey stratum, given its proximity to the targeted crop, has considerable potential for habitat manipulation through the introduction of vegetation cover. The cover may be established by allowing wildflowers and grasses to grow or by sowing selected plant species. Its positive contribution to increasing the abundance and diversity of natural enemies on crop canopies has been observed in many studies (Danne et al., 2010; Nicholls et al., 2000; Silva et al., 2010; Thomson and Hoffmann, 2009), and this habitat management practice has been seen as a promising option for IPM optimization in fruit orchards (Rieux et al., 1999). However, the effects of understory habitat management are complex and depend on a number of factors such as crop type and vegetation cover composition. Some studies have found that understory habitat management does not affect natural enemy abundance in crops (Bone et al., 2009; Bugg et al., 1991) and even observed some negative effects in relation to pest suppresion (Simon et al., 2010).

Green lacewings (Neuroptera: Chrysopidae) are predators of numerous soft-bodied insects, mites and eggs that are known to play a biological control role by consuming insect pests in almost all agricultural ecosystems (Duelli, 2001; Stelzl and Devetak, 1999). Chrysopids have a complex life history. While all larvae are predaceous, adults of most species rely on vegetal substrates for feeding and are believed to be attracted to large patches of flowering plants

(Villenave et al., 2006). Although vegetation cover may therefore potentially contribute to increasing this group of predators, few studies have confirmed their positive impact on lacewing densities in the perennial crop canopy (Daane, 2001). In southern Spanish olive orchards, green lacewings, mainly those of the *carnea*-group, are known to play a predominant role in the predation of eggs of the carpophagous generation of the olive moth *Prays oleae* (Bernard) (Campos, 2001; Szentkirályi, 2001b). Early predatory consumption of these eggs prevents the pest from causing fruit fall, which is the most important effect attributed to this key pest in olive cropping. Some years, predatory efficiency may result in a drastic decrease in the high rate of olive moth attacks on protected fruits (Ramos and Ramos, 1990).

Nowadays, the predominant understorey management strategy in Spanish olive orchards consists of maintaining a bare, weed-free soil all year round by using a combination of herbicides and minimum tillage or by using herbicides alone (ESYRCE, 2009). Nutrient and soil loss occurring under this type of soil management is progressively driving farmers, encouraged by local authorities, to introduce vegetation cover, which is known to be highly effective in preventing the severe erosion problem mentioned above (Gómez et al., 2009). To date, 22.4% of the total area occupied by Spanish olive orchards has been managed using vegetation cover in the form of resident weedy plants (ESYRCE, 2009). In this context, the objective of our study was to determine the impact of this soil management practice on the abundance and diversity of green lacewings and their availability for the biological control of *P. oleae*.

7.3 Materials and methods

7.3.1 Study site

The experiment was conducted in a 235 ha commercial olive orchard (37º 17' 46.7" N, 3º 46' 28.7" W) situated in a large, homogeneous, intensively farmed olive-growing area 20 km northwest of the city of Granada in southern Spain. The climate is typically Mediterranean, with a local annual mean temperature of 16.2ºC and an average annual rainfall of 438.3 mm (average of data for 2001 to 2010). The orchard, managed according to integrated growing

regulations for fertilization, soil management, weed control and plant protection, was planted with 90-yr-old trees exclusively of the 'Picual' cultivar, grown 10×10 m apart (at a density of 110 to 130 trees per ha^{-1}) without irrigation. The trees were pruned every other year. The surrounding landscape consisted of olive plantations of a similar structure except for a field of commercial wheat located near a stream on the eastern edge of the orchard used in our experiments (Fig. 1). The deep, well-drained, Calcaric Calcisol soil is, according to the IUSS Working Group WRB (W.R.B., 2006), typical of olive orchards in southern Spain.

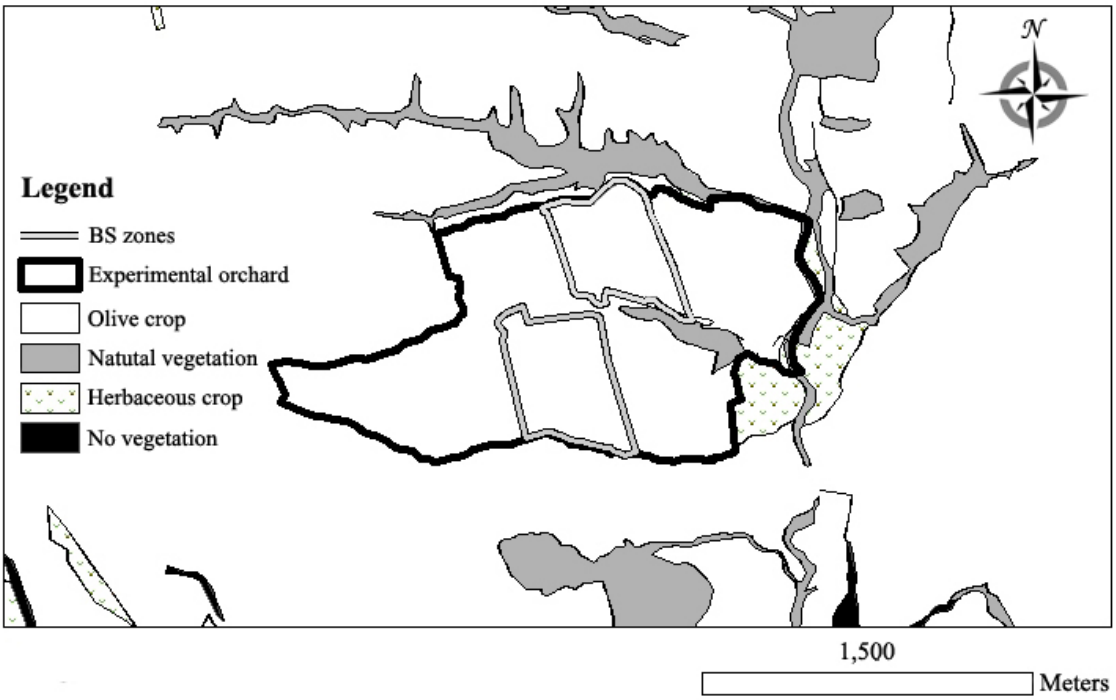

Figure 7.1. Experimental orchard and BS zones location and surroundings land use.

Soil management involved maintaining weed cover situated outside the canopy which grows in over 1.5 m-wide strips perpendicular to the slope direction. No mechanical or herbicidal control was applied to this cover, which was allowed to progressively senesce during the month of June. The strips of bare soil under the canopies were maintained by means of 4 L ha^{-1} Oxifluorfen 24% chemical herbicide in a 400 L ha^{-1} solution for preemergence treatment in late winter, and Gliphosate 36 % whenever further patch postemergence applications were required. Additionally, chopped pruning residues were used to create inert intercrop cover strips, running along the direction of the slope and perpendicular to the weed cover strips. Thus, no weeds were present on

the inert cover strips. The orchard had been under the same soil management system for over 10 years. During the study, no fertilizers, insecticides or other pest control methods were employed.

Two different soil treatments were compared during the experiment: (1) maintenance of long-established resident cover during the usual period, hereafter referred to as *vegetation cover* (VC), and (2) a treatment without vegetation cover between the rows of trees, hereafter referred to as *bare soil* (BS). During the years 2009 and 2010, the VC zones were managed as usual. The BS zones were chemically mowed, as described previously, to remove weeds from the entire soil surface (both under the tree canopy and between the rows of trees). BS treatment covered an area of 54.4 ha divided into a northern and southern zone (Fig. 1) by a gravel road alongside a gully running in an east-west direction.

7.3.2 Sampling methods

7.3.2.1 Chrysopids

Lacewing adults in both VC and BS were monitored using McPhail traps, which are widely employed for lacewing sampling in olive groves (Corrales and Campos, 2004; Szentkirályi, 2001b). The traps were baited with an aqueous solution of 5% diammonium phosphate as an attractant plus 2% Borax® for insect conservation and were suspended facing north at a height of between 1.5 and 2.0 m on the inner side of the trees. Forty McPhail traps were displayed in plots, each containing five traps. Traps within each plot were placed at least 20 m apart. Plots were replicated up to four times (20 traps) per treatment and zone and were always placed on north-facing slopes and positioned at least 150 m apart within the same zone and 750 m apart between the northern and southern zones. Captures were examined biweekly from late March to September in 2009 and 2010, and their contents were filtered using a nylon mesh to obtain the adult lacewings.

In addition, canopy suction samples were obtained using an insect aspirator (Modified CDC Backpack Aspirator Model 1412, John W. Hock Co., Gainsville, FL, USA) to determine larval and adult abundance associated with

olive trees. Square plots consisting of 16 trees (4×4) were sampled according to the method described by Porcel *et. al.* (2011). In 2009, three plots were sampled per treatment in May, June and September, two in the northern zone of the orchard and one in the southern zone (a total of 96 trees) . Suction plots were placed at least 100 m apart within each zone and 500 m apart in the area between the zones and were always north-facing. In 2010, due to an inadequate level of captures in 2009 (Table 1), the number of suction samples was increased to six plots per treatment and per zone (192 trees) and were obtained every 10 days between March and September. On arrival from the field, all samples were frozen for insect conservation purposes. In the laboratory, the lacewing larvae and adult in the suction samples were separated from other insects and vegetal material. The adults captured in McPhail traps were also separated from the rest of the insects. All the individuals were refrozen for later identification under a stereomicroscope (Stemi SV8; Zeiss, Oberkochen, Germany) following instuctions provided by the taxomonic specialist Dr. Víctor Monserrat (Complutense University, Madrid). Adults were identified up to species level and larvae to genus level. Dr. Víctor Monserrat also clarified issues regarding the identification of uncommon species.

7.3.2.2 Prey presence

The presence of *P. oleae* eggs laid on the olive fruits, chrysopidae's principal prey in olive trees, was monitored biweekly on three consecutive sampling occasions; the first sampling was carried out when the fruit was formed and was receptive to *P. oleae* oviposition. Two olive frutis were selected from a random selection of 10 olive twigs (20 olives per tree). Plots, consisting of five trees (100 fruits) and replicated four times per treatment and per zone (400 fruits), were at least 20 m apart within each zone and 750 m apart in the area between the northern and southern zones. The eggs were counted under a stereoscope and expressed in eggs per tree.

7.3.2.3 Plants

The plant density and species composition of VC were estimated by measuring 20 m linear transects along the VC strips. Measurements were

carried out annually in 2009 and 2010 at peak standing VC in early June. In 2009, 15 transects were conducted in the southern zone of the orchard and 24 in the northern zone. In 2010, 18 transects were conducted in the southern zone and 13 in the northern zone. The point-intercept method was used for each transect by driving 20 sharpened pins (1 m long, 5 mm ø) into the ground 1 m apart, making sure that the pin protruded above the vegetation. At each position, the number of times the pin touched each plant species was recorded. VC density was calculated as the number of total plant contacts divided by the total number of points measured.

7.3.3 Statistical analysis

7.3.3.1 McPhail traps

Total abundance and species diversity of chrysopid adults captured in McPahil traps were analyzed using a generalized additive mixed model (GAMM). Additive models are non-parametric extended versions of linear models that can process a mix of parametric and non-parametric variables (Yee and Mitchell, 1991) allowing for non-linear relationships between the response variable and all or some of the explanatory variables. The relationship between these variables is thus modeled using non-parametric smoothing curves (Zuur et al., 2009). Additive modeling is a useful tool for modeling abundance trends as a non-linear function of time (Fewster et al., 2000). We used the GAMM to model the temporal variability of abundance and diversity. Smooth functions were constructed using a penalized regression spline approach, and the amount of smoothing was automatically estimated by cross-validation (Wood, 2006). During data analysis (Zuur et al., 2010), the autocorrelation function (ACF) plots for regularly spaced time series revealed the existence of serial dependence of observations over time. To account for this temporal dependence, we took advantage of the GAMM's ability to deal with fixed and random effects and added an auto-regressive model of order 1, AR(1), to the GAMM as a random effect to allow for the temporal correlation. Akaike's Information Criteria (AIC) were used to verify whether the inclusion of the dependence structure improved the models by comparing their fit with and without the correlation structure. AIC provide an effective technique for selecting the best approximating model for data analysis (Burnham and

Anderson, 2002). Two GAMMs were constructed with abundance and diversity as response variables. Species diversity was calculated using Shannon's index for captures per McPhail trap and sampling date. This index takes into account species richness and evenness, with 0 representing entirely skewed species dominance and 1 representing perfectly even relative abundances. In relation to the design of the experiment, to achieve the highest level of homogeneity, we selected an orchard which, though quite large, operates under similar agronomical and environmental conditions. However, during the preliminary data analysis stage, we noticed spatial heterogeneity between data from the plots in the northern and southern zones. Zone was therefore included as a variable in the models in order to improve fit. Thus, both models use treatment (VC–BS), zone (north - south) and year (2009–2010) as categorical variables and sampling date as a smooth function. To construct the models, we used the time series data from the first sampling date in late March until VC was completely dry in the month of July. Chrysopid abundance count data was modeled using a Poisson distribution and the log link function. Overdispersion was corrected using a quasipoisson model. A Gaussian distribution was selected for the species diversity GAMM using an identity link function. Homoscedasticity and normality assumptions were verified using graphical representations of the model residuals versus fitted values and the residuals histogram, respectively (Zuur et al., 2007).

Principal response curves (PRC) were used after univariate analysis in order to study the temporal effect of VC on the chrysopid community and to identify the individual contributions of each species to differences between treatments. PRC is a multivariate technique for assessing species communities and is an appropriate tool for studying the effect of treatments referenced to a control over time. PRC are based on partial Redundancy Analysis (pRDA), a more restricted version of Principal Component Analysis (PCA) (Van den Brink and Braak, 1999). This type of analysis only extracts information from the variance defined by the explanatory variable (VC–BS) and time variable (sampling date) tested and focuses on the difference between the species composition of the treatment and the control at the corresponding time (Moser et al., 2007). The analysis is presented graphically in a diagram that plots the canonical coefficient (c_{dt}) relative to the control on the y-axis against time on the x-axis. The same graph, plots species weight (b_k), representing the

importance of each species for the response given in the diagram. High b_k values indicate that the response of the species is likely to follow the pattern in the PRC diagram; low b_k values of between –0.5 and 0.5 indicate a weak response while high negative values show a reverse trend from that in the PRC diagram. Separate PRC analyses were carried out for each year (2009 and 2010) and covered the whole sampling period (March to September). Monte Carlo permutation tests were conducted to test the significance of the y-axis.

7.3.3.2 Suction samples

Each statistical sampling unit consisted of the sum of all chrysopids collected from the square plots containing 16 trees. Adult suction data from 2009 was analyzed using a generalized linear model (GLM) with a negative binomial distribution error due to over-dispersion and the log link function. Treatment and the two sampling dates were tested as categorical variables. Zone could not be included as no replications were carried out in the southern zone. The collection of larvae in 2009 was insufficient to construct an adequate model and was therefore not tested statistically. 2010 suction data for adult and larval abundance was analyzed using GAMMs as described for McPhail trap captures. The AR-1 model was added as temporal dependence was also detected. Biodiversity was not analyzed due to the limited number of the different species collected through suction during VC presence. Adult and larva changes in the community structure were analyzed using PRC multivariate analysis as described above.

7.3.3.3 Prey and plants

The number of eggs deposited by *P. oleae* on olive fruits was analyzed using a generalized linear mixed model (GLMM) with a log link and a negative binomial error distribution due to overdispersion. The model used treatment, zone and year as categorical variables and sampling date modeled as a random effect.

VC density was compared between zones and years using two-way analysis of variance (ANOVA).

7.4 Results

7.4.1 McPhail traps

Table 1. Abundance and proportion of each green lacewing adult species captured in McPhail traps and suction samples.

Taxonomic status[a]	2009 (Traps)		2010 (Traps)		2010 (Suction)	
	Abund.	%	Abund.	%	Abund.	%
Gen. *Chrysoperla* Steinmann, 1964						
C. carnea s. l. (Stephens, 1836)	799	58.1	1,129	54.1	420	79.7
C. mediterranea (Holzel, 1972)	0	0.0	6	0.3	0	0.0
Gen. *Dichochrysa* Yang, 1991						
D. prasina (Burmeinster, 1839)	377	27.4	750	35.9	99	18.8
D. flavifrons (Brauer, 1850)	71	5.2	117	5.6	2	0.4
D. picteti (McLachlan, 1880)	45	3.3	6	0.3	0	0
D. subcubitalis (McLachlan, 1880)	2	0.1	2	0.1	1	0.2
D. genei (Rambur, 1842)	2	0.1	6	0.3	0	0.0
D. venosa (Rambur, 1842)	0	0.0	1	0.0	0	0.0
Gen. *Chrysopa* Leach in Brewster, 1815						
C. formosa Brauer, 1850	0	0.0	15	0.7	3	0.6
C. viridiana Schneider, 1845	5	0.4	4	0.2	2	0.4
Gen. *Rexa* Navás, 1919						
R. lordina Navás, 1919	70	5.1	45	2.2	0	0.0
Gen. *Cunctochrysa* Hölzel, 1970						
C. baetica (Hölzel, 1972)	3	0.2	3	0.1	0	0.0
Gen. *Suarius* Navás, 1914						
S. walshingami Navás, 1914	0	0.0	3	0.1	0	0.0

[a] All species belong to Subfamily *Chrysopinae* Esben-Petersen, 1918, Trib *Chrysopini* Schneider, 1851.

Table 2. GAMMs results. The estimate, standard error (SE), *t* value, *P*-value and degrees of freedom (df) are reported for the categorical variables. *F* statistic, *P*-values and df are reported for the smoothed variables.

Model	Variables	Estimate	SE	*t*	*F*	df	*P*-value
McPhail cpt	Treatment (VC)	0.404	0.073	5.55		1	< 0.001
	Zone (south)	0.638	0.077	8.30		1	< 0.001
	Date (2009)				24.43	4.52	< 0.001
	Date (2010)				285.85	4.42	< 0.001
Shannon index	Treatment (VC)	0.112	0.042	2.64		1	0.008
	Date (2009)				17.02	4.71	< 0.001
	Date (2010)				11.73	4.71	< 0.001
Suction adults[a]	Treatment (VC)	0.809	0.181	4.43		1	< 0.001
	Zone (south)	1.258	0.204	6.16		1	< 0.001
	Date (2010)				17.76	6.34	< 0.001
Suction larvae[a]	Treatment (VC)	0.544	0.153	3.56		1	< 0.001
	Date (2010)				16.32	6.07	< 0.001

[a]Models constructed only for samples taken in 2010.

A total of 3,461 adult chrysopids were captured in McPhail traps during the whole sampling period. Nine species from 5 genera were captured in 2009 and 12 species from 6 genera in 2010 (Table 1). In both years, *Chrysoperla carnea s.l.* and *Dichochrysa prasina* (Burmeister) were the most abundant species, followed by *Dichochrysa flavifrons* (Brauer) and *Rexa lordina* Navás. The other species accounted for 4.1% of the total captures in 2009 and 1.8% in 2010. The species *Suarius walshingami* Navás was captured for the first time in an agricultural site.

During the presence of VC, captures totaled 2,124 individuals and were considerably more abundant in VC plots, with 3.50 ± 0.25 (mean ± SE) compared to BS plots, with 2.36 ± 0.18 (Table 2). Capture rates in VC plots were higher on almost all the sampling dates (Fig. 2); the differences were more accentuated in late May of 2009 and early June of 2010, coinciding with the modeled flying peaks (Fig. 3 a and b). The number of captures was also significantly lower in plots situated in the northern zone (2.03 ± 0.15) than in

those in the southern zone (3.83 ± 0.26). No difference in abundance was observed between years, as the year variable was not significant in the model.

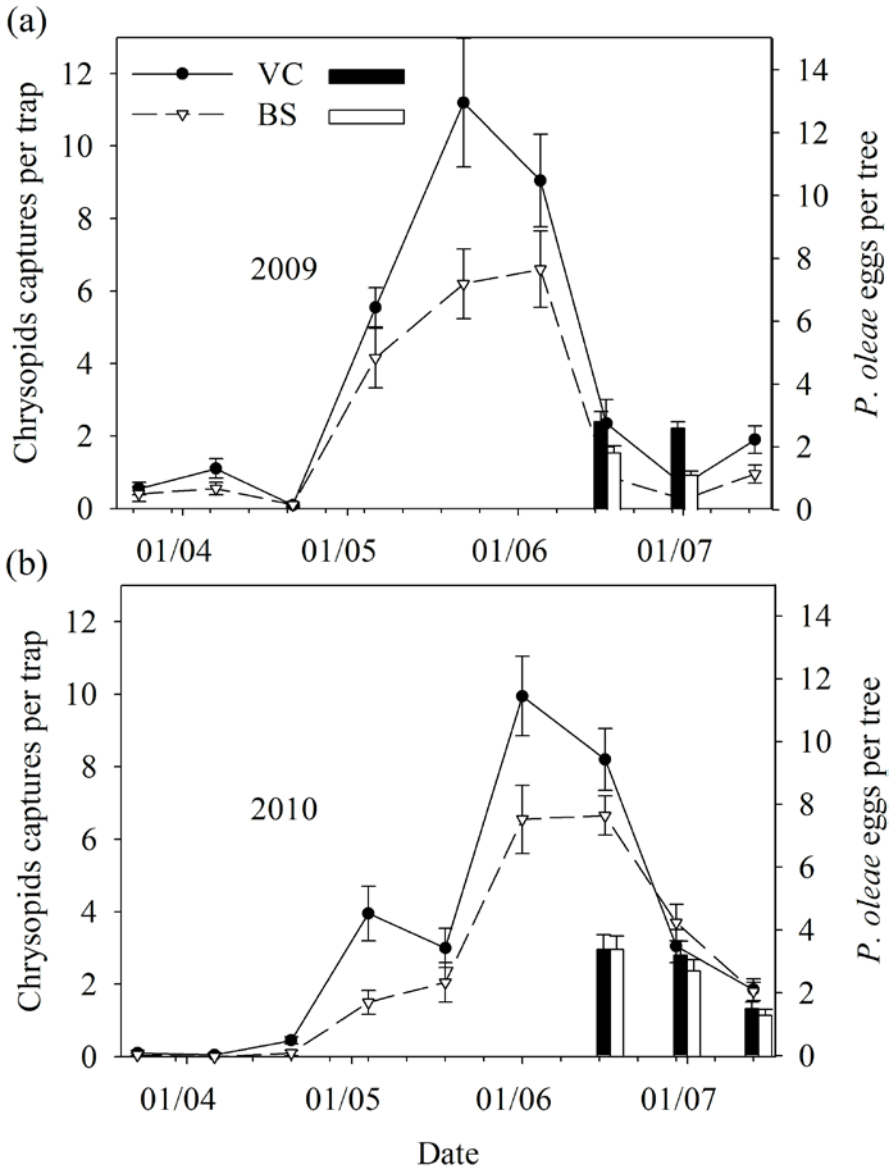

Figure 7.2. Mean chrysopid adults captures per McPhail trap (± SE) in VC and BS plots during the presence of the VC (line plot) and mean number of *P. oleae* eggs per 20 fruits collected per tree in VC and BS plots (horizontal bar chart) in the years (a) 2009 and (b) 2010

The Shannon index was also significantly higher in VC plots than in BS plots (Table 2), with values of 0.48 ± 0.03 and 0.40 ± 0.03 (mean ± SE), respectively, during the presence of VC. Capture diversity was higher on almost all the sampling dates in 2009 (Fig. 4 a) and during the month of June in 2010 (Fig. 4 b). Chrysopid diversity peaks were detected in May 2009 and June 2010, which roughly coincide with maximum abundance levels in both

these years (Fig. 3 a, b, c and d). Zone and year were not found to influence diversity.

PRC analysis of McPhail captures revealed species composition deviance in the VC treatment as compared with BS which showed a similar seasonal pattern in both 2009 and 2010 (Fig. 5). Important positive deviations were observed during the presence of VC in May and June. From the end of June onwards, low or negative deviances were observed until the month of September. The species *C. carnea s.l.* and *D. prasina* were the major contributors to the differences detected in the years 2009 and 2010 (Fig. 5). The species *D. flavifrons* and *Dichochrysa picteti* (McLachlan), captured in relatively high numbers during VC presence, were not observed to contribute to the variations caused by the treatment. Such was the case for *R. lordina*, captured only in May and early June, which was also abundant in BS plots. *Dichochrysa subcubitales* (McLachlan), *Cunctochrysa. baetica* (Hölzel), *Chrysopa. viridiana* Schneider, *Chrysopa formosa* Brauer, *Chrysoperla. mediterranea* (Hölzel) and *S. walshingami* were observed almost exclusively from late July until the end of the sampling period (outside the VC season) and therefore did not affect the deviances obtained. Small numbers of the species *Dichochrysa venosa* (Rambur) and *Dichochrysa genei* (Rambur) were captured (Table 1). All the species listed were captured on at least one occasion under both VC and BS conditions.

7.4.2 Suction samples

In 2009, 67 individual adult chrysopids were obtained by suction on the two dates sampled. A mean ±SE of 5.66 ± 1.33 individuals was collected in VC and 5.50 ± 2.71 individuals in BS plots, with no difference being observed between the treatments (GLM, $t = 0.03$, $P = 0.975$) or between sampling dates (GLM, $t = 1.10$, $P = 0.302$). A total of 16 larvae were recorded in 2009, distributed evenly between VC and BS plots (8 individuals from each). Only three individual larvae were observed in May, which increased to 13 in June. A statistical comparison of the data was not made due to the small number of individuals observed. All the adults collected in 2009 belonged to the species *C. carnea s.l.* and the larvae to the genus *Chrysoperla*.

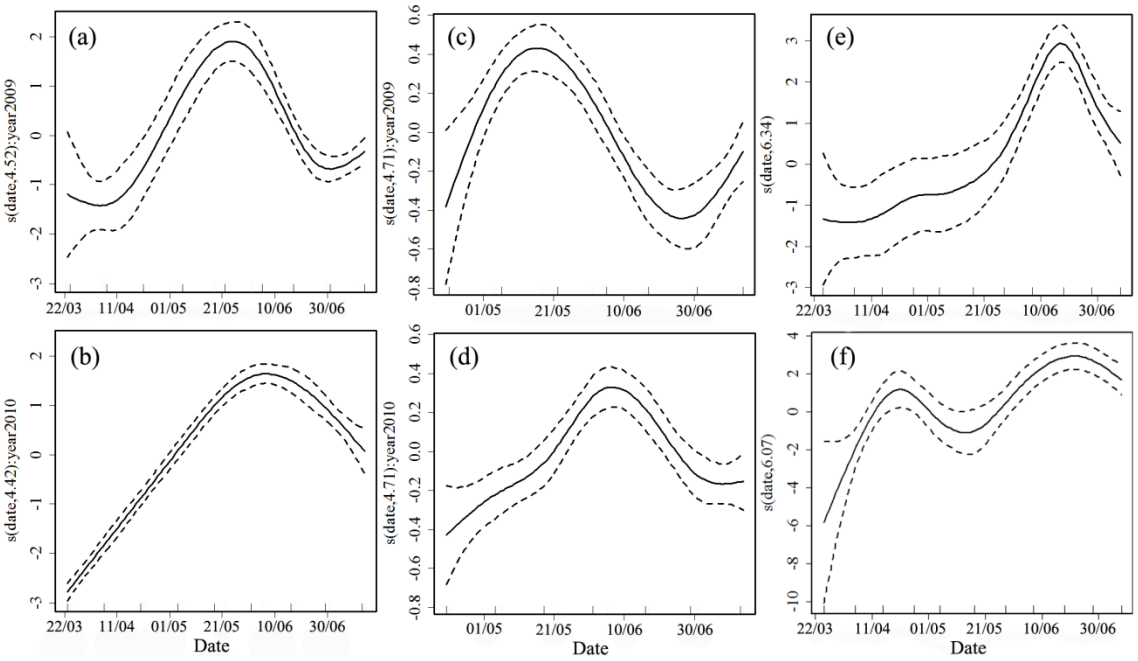

Fig. 7.3. GAMM smoothers with 95% pointwise confidence bands representing the influence of the sampling date variable on chrysopid adult captures per McPhail trap in (a) 2009 and (b) 2010. Shannon diversity index per McPhail trap in (c) 2009 and (d) 2010 and chrysopid (e) adults and (f) larvae collected per plot in 2010 through suction sampling. The estimated degrees of freedom are shown in the y-axis label. Smoother *P*-values are shown in Table 2.

In 2010, due to the increased sampling effort described above, a total of 527 individual adults from 5 species and 3 genera were captured between March and September (Table 1). As was also the case for McPhail traps, *C. carnea* s.l. and *D. prasina* were the predominant species collected by suction, although a smaller number of *D. prasina* was obtained as compared to trap captures (Table 1). The number of larvae sampled also increased sharply compared to 2009, with 513 individuals being recorded. Two genera, *Chrysoperla* and *Dichochrysa*, accounted for 49.9% and 50.1% of total larvae, respectively. A total of 236 adults and 176 larvae were collected during the VC presence period. Adults were sampled from olive trees in considerable numbers during June (Fig 6) and peaked in mid-June, which is later than the capture maximum registered for trapping (Fig. 3 b). Adult abundance was significantly higher in VC plots (Table 2) which averaged 2.47 ± 0.73 (mean ± SE) compared to 1.11 ± 0.36 in BS plots. VC plots showed higher abundance values on all the sampling dates during the month of June (Fig. 6 a). Significantly fewer adults were obtained in the northern zone (0.78 ± 0.17) as compared to the southern zone (2.78 ± 0.34) (Table 2). Larvae displayed a bimodal pattern and peaked twice (in mid-April and mid-June) (Fig. 3 f). The

highest abundance registered in June coincided with the presence of adults in olive canopies (Fig. 3 e and f). As with the adults, larvae abundance in VC plots (1.68 ± 0.32, mean ± SE) was significantly higher than in BS plots (0.98 ± 0.22) during the VC presence period (Table 2). No zone effect was detected for larvae abundance despite the lower values observed in the northern zone (1.21 ± 0.23) as compared to the southern zone (1.45 ± 0.32).

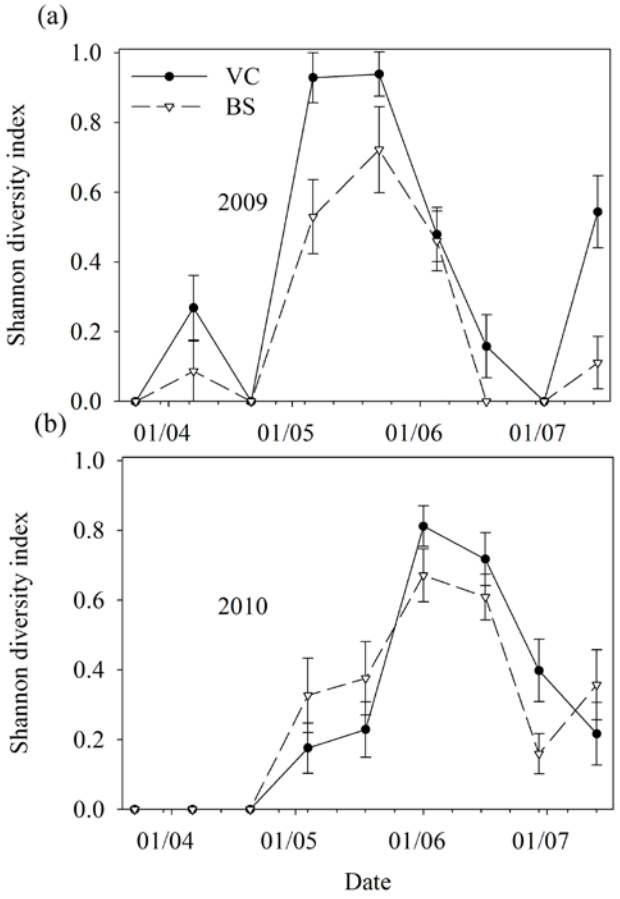

Figure 7.4. Shannon diversity index per McPhail trap (mean ± SE) in VC and BS plots during the presence of the VC in (a) 2009 and (b) 2010.

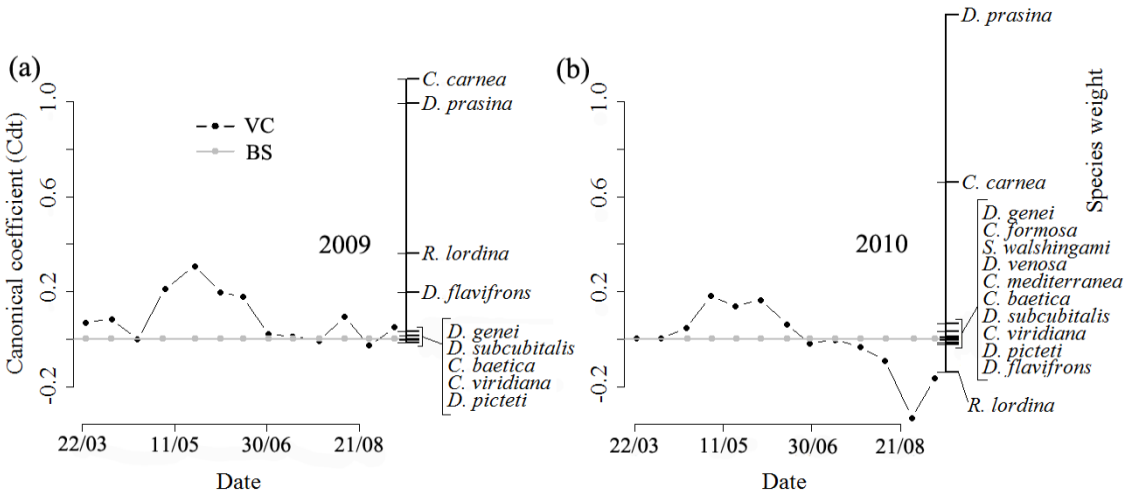

Figure 7.5. PRC diagrams showing the effect of VC relative to the control (BS) on chrysopid species composition of McPhail captures per trap throughout the sampling period in the years (a) 2009 and (b) 2010. The BS control is represented by zero deviance on the y-axis. The numerical scale on the left y-axis applies to both the canonical coefficients (c_{dt}) represented in the line plot and the species weight (b_k) indicated for each species on the right y-axis. The x-axis of both models was significant (Monte Carlo permutation test, $P < 0.01$).

PRC analysis of adults showed positive deviations from the BS treatment during the month of June (Fig. 7 a) when the highest abundance rates and greatest differences in total adult abundance between treatments were registered (Fig. 6 a). Small or negative deviations were obtained in July, August and September. *C. carnea s. l.* was the only species to contribute to the deviations observed, as only 10 *D. prasina* individuals (4.23% of the total) were collected in the olive canopy during VC presence. The other species appeared between July and the end of the sampling period. PRC analysis of larvae showed sustained positive deviations from the beginning of June until the end of July, beyond the VC presence period (Fig. 7 b). Again, only larvae of the genus *Chrysoperla* contributed to the difference observed as they were much more abundant than *Dichochrysa* larvae (22.1%) from March to late June.

7.4.3 Prey

As olive fruit becomes receptive to *P. oleae* antophagous generation during mid-June in both 2009 and 2010, *P. oleae* egg samples were taken between mid-June and mid-July on three occasions (Fig. 2). The number of eggs recorded on the two sampling dates in June 2009 was similar. However, no eggs were present in July (Fig. 2 a) since olives already exceeded the

permissible size for olive moth oviposition. In 2010, as in the previous year, eggs were present during the second half of June, although, unlike in 2009, oviposition, though declining, continued into mid-July (Fig 2 b). The number of olive moth eggs was higher in VC plots in 2009 (Fig. 2 a), which was not the case in 2010 (Fig. 2 b). Overall, the differences were only significant to a 90% confidence level (GLMM, $t = 1.75$, $P = 0.081$) possibly due to the large deviance observed. The *P. oleae* attack rate was higher in 2010 compared to 2009 as year was the only significant variable in the model (GLMM, $t = 3.88$, $P < 0.01$). No difference was observed between zones (GLMM, $t = 1.24$, $P = 0.214$).

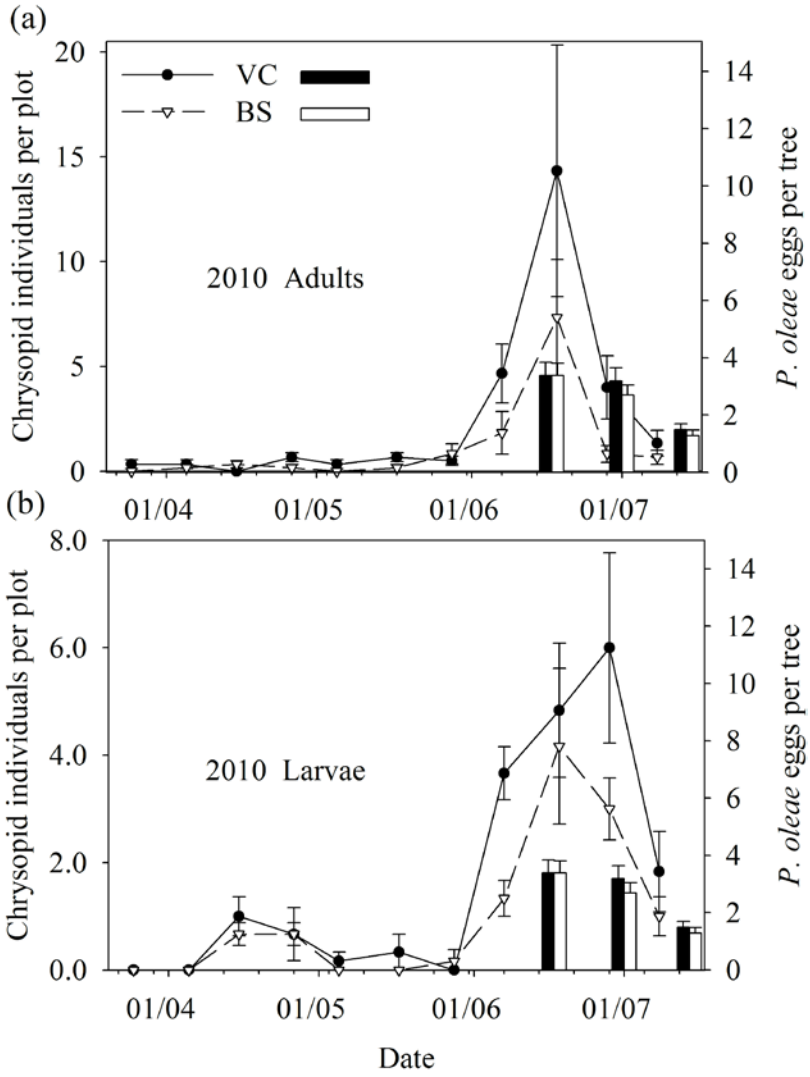

Figure 7.6. Chrysopid (a) adults and (b) larvae collected per plot through suction sampling in VC and BS plots during the presence of the VC (line plot) and mean number of *P. oleae* eggs per 20 fruits collected per tree in VC and BS plots (horizontal bar chart) in the year 2010.

Chrysopid adults caught in McPhail traps started to build up and reached their maximum levels before the onset of olive moth oviposition in both 2009 and 2010 (Fig. 2). As for their presence in the olive canopy recorded through suction sampling in 2010, maximum adult abundance showed temporal correspondence with the onset and maximum presence of *P. oleae* eggs (Fig. 6 a). The peak in the larvae population detected on olive trees in June also coincided with prey presence (Fig. 6 b).

7.4.4 Plants

The Poaceae species *Bromus madritensis* L. *Lolium rigidum* Gaudim and *Hordeum leporium* Link dominated the indigenous VC (Fig. 8). Flowering plants represented 29.7% of the total recorded, with *Anacyclus clavatus* Reichenb (Asteraceae) and *Medicago minima* (L.) (Fabaceae) being the most abundant. Total VC density reached values of 1.52 ± 0.11 (mean ± SE) in 2009 and 1.63 ± 0.09 in 2010, with no statistical difference being observed between years (ANOVA, F = 0.33, df = 2, P = 0.567). Also, no difference in VC density was observed between zones (ANOVA, F = 1.22, df = 2, P = 0.273), with values of 1.49 ± 0.09 recorded in the northern zone and 1.65 ± 0.12 in the southern zone.

7.5 Discussion

The importance of natural vegetation patches for lacewing concentrations and conservation has been reviewed by Szentkirályi (2001a), who identified valuable ecological structures such as hedgerows, flower strips and ground cover. Despite the important role played by natural vegetation in green lacewing diversity and conservation, little evidence of the function of ground cover in increasing lacewing presence on the targeted crop has been found in orchards. Smith et al. (1996) registered 3 to 5-times higher *Chrysoperla rufilabris* (Burmeister) abundance in the month of July in pecan trees with legume cover as compared to those with grass cover. Similarly, in apple trees, Wyss (1995; 1996) recorded larger numbers of *C. carnea s.l.* individuals associated with weedy strips compared to rye grass cover for all three sampling methods employed. Although these findings are consistent with the results presented in our study, similar studies have not observed this impact of cover. In vineyards, neither grass nor saltbush VC influenced the

presence of lacewings on vine canopies, even though positive results were observed for predators (Danne et al., 2010); in apple orchards, a mix of selected plants failed to increase green and brown lacewing abundance on tree canopies (Bone et al., 2009).

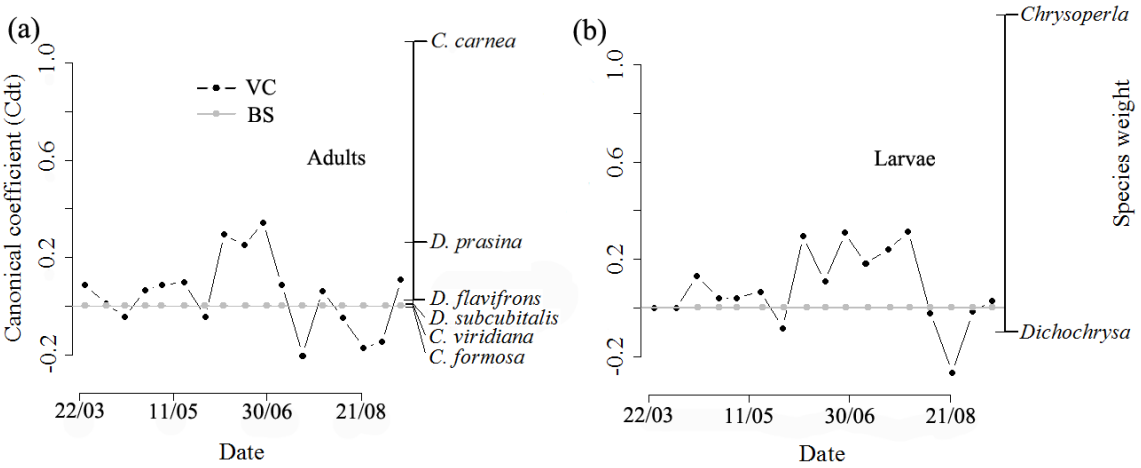

Figure 7.7. PRC diagrams showing the effect of VC relative to the control (BS) on chrysopid (a) adult and (b) larvae species composition collected per plot through suction sampling throughout the sampling period in the year 2010. The BS control is represented by zero deviance on the y-axis. The numerical scale on the left y-axis applies to both the canonical coefficients (c_{dt}) represented in the line plot and the species weight (b_k) indicated for each species on the right y-axis. The x-axis of both models was significant (Monte Carlo permutation test, $P < 0.01$).

This discrepancy could be explained by various factors. In general, lacewing individuals were collected in small and even insignificant numbers as reported by Bone et al. (2009). Indeed, studies aimed at studying the effects of ground cover and cover crops rarely consider chrysopids in isolation from other predators, meaning that sampling efforts and methods are not adapted to chrysopid detection requirements. In this regard, Silva et al. (2010), who compared the effect of two types of VC with a BS control in 2003, observed no significant differences between treatments using the beating method. However, using suction sampling (carried out simultaneously), over three times more lacewings were obtained, and important differences in the canopy between VC treatments and the control were observed. In our study, the small size of the sampled population could also explain why no difference in the number of adults collected by suction was observed in 2009 unlike the marked differences recorded in the same year by McPhail trapping.

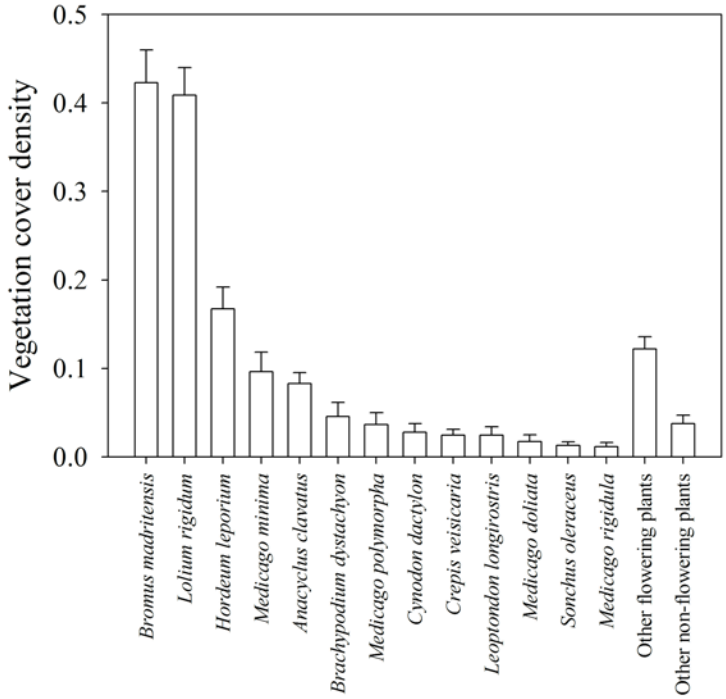

Figure 7.8. Mean cover density (± SE), expressed in plant contacts per pin in 20 pins transects (20 meters), of the 13 most abundant plant species and rest of the flowering and non-flowering species of the resident VC. Up to 71 different vegetal species were identified in the VC.

The size of the experimental plots may also be an important factor. Prasifka et al. (2005) have observed effects of scale on lacewings and have suggested that abundance evaluation experiments carried out on small plots could not be regarded as equivalent to those conducted on a field-size scale. Most of the agriculturally important lacewing species are known to cover large distances due to their constant nomadism (Duelli, 2001). Thus, small scale plots may be affected by rapid lacewing colonization from the immediate surroundings. Certainly, lacewing abundances evaluation must therefore benefit from experiments designed for larger scales, which is consistent with the positive results reported by Smith et al. (1996) using 10 ha experimental plots and those described in our study (plots of over 20 ha).

Another factor that might explain the differences between the studies is the timing of VC senescence. Bone et al. (2009) reported that an increase in natural enemies in the cover crops did not necessarily influence numbers in the apple tree canopy. Non-crop plants may arrest egg-laying females if the prey presence period overlaps that of the crop (McEwen and Ruiz, 1994; Szentkirályi, 2001a). Interestingly, Smith et al. (1996) found that legume VC

improved *C. rufilabris* presence on pecan trees just after VC senescence during the month of June. Our 2010 results also show that maximum differences between VC and BS treatments for adults and larvae collected directly from the olive canopies by suction occurred in mid-June, coinciding with the decline in VC. Furthermore, the temporal distribution of McPhail adult catches and those collected in suction samples did not coincide in 2010. Abundance levels registered through trapping started to build-up and peaked earlier than presence on tree canopies. Traps, though hung on the inner side of the trees, may have attracted green lacewings at that time associated with VC. This would also be in line with the possible movement of chrysopids from VC to tree canopies during the VC senescence period, suggesting that the presence of VC may act as a reservoir that increases lacewing activity in the crop after VC disappears. The change in the vegetal substrate in olive groves may also be boosted by the presence of *P. oleae* eggs, one of the most abundant types of prey in olive trees, as indicated by the extraordinary temporal coincidence observed with adult abundance on trees.

Apart from the effect of VC, unexpected differences in adult abundance were recorded between zones, suggesting the existence of an important unmeasured factor in the experiment. The reason for this is unknown and cannot be explained by the VC heterogeneity or prey availability as shown in our results. The existence of a large forest stand located about 1 km away and close to the southern zone, may be responsible for increasing chrysopid occupancy in this area.

Our results show that VC slightly increased chrysopid biodiversity but did not alter species assemblages. VC therefore increased species evenness by reducing *C. carnea s. l.* dominance in VC plots. It has been observed that lacewing species richness and diversity in agricultural fields are determined by the heterogeneity and quality of adjacent habitats (Szentkirályi, 2001a), although, to date, no effect of understorey habitats has been reported. The limited impact of VC further confirms that green lacewing species assemblages in agricultural fields are related to environmental factors on a larger scale. In general, species richness was quite high in relation to that expected for olive groves (Szentkirályi, 2001b) which can be attributed to the absence of

insecticidal treatment that negatively impacts species richness and diversity (Porcel et al. unpublished data).

The most abundant species captured in McPhail traps were the most important cause of the differences observed between VC and BS. Both *C. carnea s.l.* and *D. prasina* are field-crop specialists adapted to living in nonspecific patchy environments and can benefit from the presence of VC. *R. lordin*a, which was only observed during the presence of VC, is, on the contrary, highly substrate-specific in olive trees (Monserrat, 2008), which explains why no impact on this species was observed. The minor contribution of *D. flavifrons* and *D. picteti* to the divergence between VC and BS can only be explained by the limited amount of captures compared to the highly similar species *D. prasina*.

Surprisingly, suction collected an almost insignificant number of *D. prasina* adults on olive canopies during VC presence. This species was not responsible for any of the difference between VC and BS at either the adult or larval stage, which may be indicative of differing behavior patterns compared to *C. carnea* s. l. *Dichochrysa spp.* appeared on olive canopies later than *C. carnea s. l.*, suggesting later displacement from VC. The temporal coincidence of *C. carnea s.l.* and *P. oleae* indicates a closer prey-predator relationship than that with *Dichochrysa spp*. Indeed, while *C. carnea s.l.* predation upon *P. oleae* has been confirmed by serological tests (Morris et al., 1999), there is no evidence on *Dichochrysa spp.*'s predatory capacity.

Green lacewing species recorded in olive orchards are mostly palyno-glycophagous (except *Chrysopa spp.*), indicating that the increase in adult abundance is mainly due to the moderate amount of flowering species present in VC. A number of studies have reported that flowering species have a positive effect on chrysopid abundance in VC compared to grass cover (Smith et al., 1996; Song et al., 2010; Wyss, 1996). Theoretically, chrysopid presence could therefore be increased by the higher densities of flowering plants. Further study is required to clarify this point. For example, Silva et al. (2010) did not observe any improvement in selected plants sown with respect to resident vegetation. On the other hand, naturally occurring climacic VC in olive orchards offers significant advantages. Implementation of this type of

habitat management is inexpensive, and disruption is cut to a minimum as sowing/resowing is not required. VC strip width, natural senescence and warm climate-adapted weed composition minimize yield loss due to competition which is critical for within crop habitats (Norris and Kogan, 2000). These advantages are likely to make growers less reluctant to adopt this habitat management practice and its implementation on a significant scale. Our study also presents evidence that resident VC in olive groves is temporally and spatially adequate to increase these valuable predators as recommended by Landis et al. (2000). VC in its most productive period coincides with the buildup in lacewing populations, and proximity to trees facilitates olive canopy colonization and availability for biological control. Resident VC may therefore be regarded as a valuable habitat manipulation resource for controlling *P.oleae* within the framework of pest-management programs.

Acknowledgements

We wish to thank Dr. Víctor Monserrat for his contribution to chrysopid identification and María Luisa Fernández Sierra and Herminia Barroso for their laboratory and field assistance. We would also like to thank the state-owned Empresa Pública para el Desarrollo Agrario y Pesquero, S.A (D.A.P.S.A.). for their cooperation and Michael O'Shea for proof-reading the manuscript. The study was funded by the Junta de Andalucía (project P07-AGR-02747).

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8. The marking of *Chrysoperla carnea* (Neuroptera: Chrysopidae) with an oil-soluble dye incorporated into an artificial larval diet

The marking of *Chrysoperla carnea* (Neuroptera: Chrysopidae) with an oil-soluble dye incorporated into an artificial larval diet

Under revision in *Journal of Economic Entomology*

8.1 Abstract

Several experiments were carried out aiming to (1) identify oil-soluble dyes, which, incorporated into a tested meridic diet for larvae, could internally mark *Chrysoperla carnea* adults, (2) detect any possible negative effects of the dye on larval survival, development, consumption rate, adult survival, fecundity and flight performance, and, on the basis of the previous information, (3) establish an adequate dye concentration for mass rearing. Among the dyes Sudan Red 7B, Solvent Blue 35 and Sudan Black B, only Sudan Red 7B produced successfully marked adults. Sudan Red 7B was tested at concentrations ranging from 0 to 1100 ppm (w/w). A relationship between mortality and dye concentration was observed. No substantial differences in development, consumption rate, fecundity and flight performance were seen across concentrations. Marked individuals showed a significantly lower adult survival rate, although they recorded quite a long life span. Dye concentrations in the diet above 450 ppm yielded marked adults identifiable simply by visual inspection. LC_{15} and LC_{20} concentrations were estimated which guaranteed an adequate marking level and an acceptable larval survival rate. This marking technique is suitable for *C. carnea* and possibly also for other chrysopids of agricultural importance.

Key words *Dichochrysa prasina*, dispersal, lacewings, Sudan Red 7B, mark-release-recapture

8.2 Introduction

Reliable marking methods are regarded as essential for the study of insect biology, ethology and demography, providing information on their movements, distribution patterns and abundance (Hagler and Jackson 2001). Beneficial insects (predators and parasitoids) are known to have a broad range of spatial scales of dispersal, and an understanding of their movement is thus essential for the three types of biological control strategies; conservation, classical biological control, and augmentation (Lavandero et al. 2004a). Chsysopid lacewings are regarded as a family of great interest due to their role as beneficial predators of pest arthropods (Stelzl and Devetak 1999). Among chrysopids, the most successful species in agricultural ecosystems are generally assumed to be highly mobile insects. *Chrysoperla carnea* (Stephens) carries out preovipository migration flights as well as continuous nomadic dispersion throughout its reproductive period in search of food sources, adequate oviposition locations and mating partners (Duelli 2001). Thus, the information provided by mark-release-recapture (MRR) experiments on *C. carnea* and other chrysopid species of agricultural importance may be useful in reaching informed IPM decisions in order to enhance biological control for crop protection.

Various marking techniques, ranging from pollen markers and externally applied dyes to protein and molecular markers, have been used to track down beneficial insects (Lavandero et al. 2004b). An ideal marking technique would be low-cost, durable, easily identifiable and would not hinder the normal development, behaviour and reproduction of insects (Southwood 1978, Hagler and Jackson 2001). Among the marking options available, the use of oil-soluble dyes incorporated into the insect's diet provides several advantages over other methods. The oil-soluble dye, a self-marking procedure which avoids the need for insect handling, is cheap, the marking is persistent over a long period of time, and no additional techniques are required to distinguish marked individuals from unmarked conspecifics (Hagler and Jackson 2001). Insects belonging to different orders have thus been labelled by using oil-soluble dyes such as Coleoptera, Lepidoptera, Diptera, Isoptera and Hymenoptera (Qureshi et al. 2004). With regard to beneficial insects,

several species of parasitoids were successfully marked by adding Acridine Orange dye to their honey diet (Strand et al. 1990). However, to date, dye marking has not been applied to other beneficial insects such as those belonging to the Chrysopidae family. This group of insects, due to their size and anatomy, could be ideal candidates for the application of this marking technique since marked individuals can be easily identified through quick direct inspection.

Recent research on internally marking insects with dyes has focused on the evaluation of the suitability of this technique for Lepidoptera species of economic importance associated with resistance-management programs (Qureshi et al. 2004, Vilarinho et al., 2006, Zhao et al. 2008). Marking efficiency depends on both the dye type and the dye concentration used in the diet, as well as the dye's possible negative biological and behavioural effects on marked insects (Hendricks 1971). Therefore, with the objective of internally marking *Chrysoperla carnea* (Stephens) adults by using oil-soluble dyes, our research aimed, firstly, to identify an effective dye marker, secondly, to assess any possible adverse effects on certain biological and behavioural parameters of the incorporated dye, and thirdly, to establish the optimal dye concentration in the diet for marking the species.

8.3 Materials and Methods

8.3.1 C. carnea Colony and Rearing Conditions

C. carnea individuals were taken from a laboratory colony established in 2005 with larvae provided by Koppert Spain (La Mojonera, Almería). Larvae were reared separately in 4.5 ø Petri dishes on *Ephestia kuehniella* Zeller (Lepidoptera, Pyralidae) purchased from Biotop (Valbonne, France) and kept in an environmental cabinet at 25 ± 1°C, 50–60% RH and for a photoperiod of 16:8 (L:D) h. Adults were transferred to rearing boxes (9.5 L), fed on an artificial diet (50% honey and 50% pollen) and supplied with mineral water, both of which were replenished once a week. The colony was replenished with a new larval stock every month.

8.3.2 Diet Preparation

A semisolid artificial diet for lacewing larvae was prepared following the methods described in Cohen and Smith (1998) for *Chrysoperla rufilabris* Burmeister and adapted and tested for *C. carnea* by Sattar et al. (2007). The ingredient ratios employed correspond to diet 2 (Sattar et al. 2007), which, according to the authors, produced the best results for green lacewing mass rearing in terms of larval survival, adult emergence and fecundity. Control, black, blue and red diets were prepared for the experiments. The oil-soluble dyes Sudan Red 7B (C.I. 26050), Solvent Blue 35 (C.I. 61554) and Sudan Black B (C.I. 26150), supplied by Sigma Aldrich (St. Louis, MO, USA), were included in artificial diets following a methodology similar to that described for dye incorporation into lepidopteran meridic diets (Ostlie et al. 1984, Zhao et al. 2008). An initial dye solution was formulated by dissolving a weighed amount of dye in 4 ml of corn oil. As the semisolid paste-like consistency of the artificial diet made it difficult to measure volume accurately, final dye concentrations in diets were calculated as a weight-weight ratio. An approximate diet volume of 23 ml was weighed, to which was added the 4 ml of corn oil used in the initial dye solution in order to obtain the final solvent weight. The amounts of dye powder necessary to achieve the final concentrations of 300, 450, 650, 800 and 1100 ppm (w/w) were individually calculated based on the final solvent weight. Finally, the dye solution was diluted in the 23 ml diet volume using a blender to obtain the final coloured diets. The control diet consisted of the basic diet plus the corn oil used as dye solvent. Two methods of supplying larvae with the artificial diet were tested. The first method involved inserting the diet between two Parafilm layers stretched to three times their original size, in order to produce flat feeding sachets (Cohen and Smith 1998). Using this wrapping method we observed that the feeding by small larvae on this flat surface was cumbersome. We therefore placed a drop of diet in the center of a single 4 × 4 cm Parafilm layer stretched to three times its size which was then wrapped around the diet, drop producing ball-shaped packets. The new packets had a round surface that proved more suitable for *C. carnea* larvae, which facilitated grasping by the penetrating mouthparts. Packets of different sizes were made in accordance with the larvae instar and ranged in weight from approximately 50 to 200 mg.

8.3.3 Marking Capacity of Blue (Solvent Blue 35), Red (Sudan Red 7B) and Black (Sudan Black B) Diets

An initial test was carried out to determine whether oil-soluble dyes could effectively mark *C. carnea* adults. A sufficient amount of less than 12 h-old recently laid eggs was taken from the adult rearing boxes and placed in 1.5 mL plastic tubes. The egg hatch was inspected twice a day. Neonate larvae (less than 12 hours old) were transferred to 4.5 ø Petri dishes in groups of 30 individuals and fed on control, blue, red and black diets at the elevated concentration of 1100 ppm. A single feeding packet was dispensed every other day until cocoon spinning began. On emergence, adults were checked for colour change directly and under a stereoscope (Stemi SV8; Zeiss, Oberkochen, Germany).

8.3.4 Effects of the Red Diet (Sudan Red 7B) on Larval Survival, Development and Diet Acceptance

The suitability of this internal marker and its optimal dose were assessed by measuring various biological parameters. The experimental larvae were obtained from the stock culture as described in the previous experiment and kept in rearing conditions inside an environmental chamber. Ten larvae per replicate were fed on a control, 350, 450, 650, 800 and 1100 ppm red diet. New feeding packets were supplied every other day. Every 12 h, the development stage and survival were recorded until adult emergence. Mortality was calculated as the proportion of deceased individuals divided by the total number of individuals per block. The development time was calculated as the number of days from the first feeding. Larvae were weighed every other day, starting on the second day after the first feeding, for 10 days by means of an electronic analytical balance (AS 220/C/2, RADWAG Balances and Scales, Radom, Poland). Pupal weight was recorded whenever moult to pupa was detected inside the cocoon (less than 12 h later), regardless of the time spent for the change in stage. Diet consumption was calculated by determining the

feeding packet weight reduction in each 2-day interval. Thus, feeding packets were weighed at the dispensing and removal stages. Both larval weight and diet consumption were estimated up to 10 days from first feeding. Only the individuals that did not die before cocoon spinning have been included in the calculations of larval and pupal weight, total and partial development times and diet consumption. The experiment was organized according to a randomized complete block design comprising five replications (50 individuals per concentration).

8.3.5 Effects of the Red Diet (Sudan Red 7B) on Adult Fecundity and Survival.

Newly emerged *C. carnea* males and females, fed throughout larval life on different concentrations of the red diet, were paired and transferred to quadrangular plastic cages (1.2 L). A self-adhesive green velvet paper (Sadipal Stationary Papers, Girona, Spain) was stuck upside-down onto the cage's removable lid which acted as an ovipositing surface. Cages were ventilated by a 5 cm ø hole covered with a thin piece of gauze. The caged adults were provided with food and water as described for the stock colony. The cages were inspected daily in order to record the number of eggs laid and adult survival. The eggs were inspected under a microscope to determine whether the F1 offspring of marked females was marked. The experiment was conducted using a completely randomized design, with the number of adults varying according to larval survival (Table 8.4).

8.3.6 Effects of the Red Diet (Sudan Red 7B) on Adult Flight Performance

The flight performance of *C. carnea* marked adults was assessed using an automated flight mill system. Individuals were reared in culture conditions on control and LC_{15} diets inside an environmental cabinet. The experimental individuals were collected during the programmed 30 min nightfall simulation in the cabinet. *C. carnea* adults are known to carry out flight activity at dawn (Duelli 1980). In total darkness, the individuals were transferred to a windowless controlled room at 22 ± 2°C and 35 ± 10% RH illuminated by a

fibre optic illuminator (DCR III, Schott, Elmsford, NY, USA) located 3 m above the arena and fitted to the ceiling providing the flight mill with a light intensity of 2 lux. The flight mill consisted of a 15.5 cm styrene rotating arm (Evergreen Scale Models, Kirkland, USA) attached to the centre of a 38 mm entomological pin as a central axle. Individuals were tethered by gluing their pronotum to a copper wire perpendicularly placed at the end of the rotating arm. To the other end, a counterweight black card was attached as a detection source for the software. The tethered adults were allowed to fly for a 5 h period. The circular movements of the mill were recorded and transmitted to the EthoVision XT system which extracted from the recorded tracks the (1) movement, (2) total distance moved and (3) mean velocity parameters. Movement indicates the duration of the flight activity as part of the total trial time, which hereafter will be referred to as the flight duration. A start/stop threshold for detection was set at 30 cm/s to avoid recording inertial movement. The experiment was carried out following a completely randomized design and considered each individual as a single replicate.

8.3.7 Data Analysis

The effect of increasing concentrations of Sudan Red 7B on the proportional number of deceased individuals was analyzed using probit analysis of binomial proportions determining LC_{15} and LC_{20} values. Development data and flight performance variables were log(x) transformed with a view to normalizing and reducing variance of the data set. Means of development, weight, larval diet consumption and flight performance variables were compared using a two-way analysis of variance (ANOVA) with diet and sex as factors. The number of deposited eggs per female was compared across treatments using a one-way ANOVA. Fisher's least significant difference (LSD) tests were performed for paired comparisons following ANOVA ($P < 0.05$). Adult survival was analysed using a Cox regression for survivorship with diet and sex as factors. The complete analysis was performed using R software for windows (R Development Core Team 2010) with functions from base packages and the survival package.

8.4 Results and Discussion

8.4.1 Marking Capacity of Blue, Red and Black Diets

During the feeding process, all three dyes changed the color of the larvae. Non-digested diet was easily observed within the insect's hind and mid-gut through the translucent body wall. After pupation, the adults that emerged from larvae fed on the red diet presented a distinguishable colour change, observable mainly in the abdomen, but present to a variable extent in the thorax and even in the head. None of the adults fed as larvae on blue and black diets exhibited color change as compared to control individuals. Generally, lepidopteran adults reared on diets containing both Sudan Red 7B and Solvent Blue 35 have yielded colour markings (Ostlie et al. 1984, Qureshi et al. 2004, Vilarinho et al. 2006). However, in accordance with our results, Sudan Blue 35 was unable to mark *Helicoverpa armigera* (Hübner) (Zhao et al. 2008). Our results confirm that the effectiveness of the blue dye is highly dependent on species and may not be an adequate insect marker. However, Sudan Red 7B, which was highly effective in marking *C. carnea,* could be quite an effective internal marker for most insects depending on its toxicity in each particular case. Since Solvent Blue 35 and Sudan Black B failed to mark *C. carnea* adults, only Sudan Red 7B was evaluated for its effects on the biology and behaviour of *C. carnea*.

Table 8.1. Mean duration (± SD) of *C. carnea* larval and pupal development fed on artificial diet with increasing concentrations of Sudan Red 7B and two-way ANOVA results.

Diet	Sex	*n*	Developmental time (days) [a]					
			1st instar	2nd instar	3rd instar	Prepupa	Pupa	Overall
Control	Both	41	3.6 ± 0.4 a	3.4 ± 0.3	3.9 ± 0.7 a	3.7 ± 0.3 ab	7.2 ± 0.5 a	21.8 ± 1.0
Red diet 300 ppm	Both	34	3.7 ± 0.4 a	3.3 ± 0.3	4.0 ± 0.5 ab	3.6 ± 0.5 a	7.1 ± 0.5 ab	21.6 ± 0.7
Red diet 450 ppm	Both	38	4.5 ± 0.7 b	3.4 ± 0.3	4.3 ± 0.7 c	3.6 ± 0.5 a	6.5 ± 0.8 c	22.2 ± 1.4
Red diet 650 ppm	Both	29	4.3 ± 0.6 b	3.4 ± 0.4	4.0 ± 0.5 abc	3.5 ± 0.4 a	6.6 ± 0.5 c	21.8 ± 1.0
Red diet 800 ppm	Both	31	3.6 ± 0.5 a	3.4 ± 0.4	4.1 ± 0.7 bc	3.9 ± 0.6 b	7.0 ± 0.8 ab	22.0 ± 1.0
Red diet 1100	Both	30	3.7 ± 0.7 a	3.6 ± 0.4	3.8 ± 0.6 a	3.8 ± 0.4 b	6.9 ± 0.4 b	21.8 ± 1.2
All diets	Female	96	3.9 ± 0.7 *a*	3.5 ± 0.4 *a*	4.2 ± 0.7 *a*	3.6 ± 0.5 *a*	6.9 ± 0.7 *a*	22.1 ± 1.2
All diets	Male	107	3.9 ± 0.7 *a*	3.4 ± 0.4 *b*	3.8 ± 0.5 *b*	3.7 ± 0.4 *a*	6.9 ± 0.6 *a*	21.6 ± 1.0
Diet (df = 5)								
F			16.64	1.78	2.33	2.41	7.33	1.29
P			< 0.001*	0.119	0.044*	0.038*	< 0.001*	0.279
Sex (df = 1)								
F			0.22	6.20	17.58	0.79	0.01	9.96
P			0.635	0.014*	< 0.001*	0.375	0.978	0.002*
Diet × Sex (df = 5)								
F			1.04	1.76	1.20	0.24	0.63	0.58
P			0.397	0.123	0.312	0.942	0.679	0.717

[a] Original data log(x) transformed for analysis of variance. Means (± SD) within the same column, followed by the same or no letter, are not significantly different, Fisher's least significance difference test ($P < 0.05$). Regular letters indicate differences in the factor diet. Italic letters indicate differences in the factor sex.

8.4.2 Survival, Development and Diet Acceptance

Larval mortality rates from 2.5 to 30.5 % were obtained for individuals fed on red diets at concentrations ranging from 0 to 1100 ppm (w/w). Some of the larvae that fed on dye-containing diets (all except the control) experienced problems with the cocoon spinning process, resulting in partially spun cocoons and, in most cases, in a lack of cocoon, leading to the death of individuals at the prepupa or pupa stages. The probit model (Fig. 8.1) indicated that an increase of the Sudan Red 7B concentration in the diet resulted in a significant increase in the mortality rate. In early experiments using this marker dye, Ostlie et al. (1984) reported significant differences in overall survival between control fed and red diet fed *Ostrinia nubilalis* (Hübner) larvae at 600 ppm. Later on, the same species was marked using a lower dye concentration of 400 ppm (w/v), which reduced mortality induced by the presence of the dye in the meridic diet to insignificant levels (Hunt et al. 2000). As in the present study, these results suggest that there is a relationship between concentration and mortality for *O. nubilalis*. Although we have observed that mortality is related to dye concentration, *C. carnea* larvae endured high concentrations of Sudan Red 7B and were able to fully develop to adulthood. LC_{15} and LC_{20} were estimated at 484.6 ppm (upper and lower 95% confidence intervals = 173.9 and 661.0 ppm, respectively) and 666.7 ppm (upper and lower 95% confidence intervals = 464.4 and 883.9 ppm, respectively).

Total development time was unaffected by dye concentration (Table 8.1). Some significant differences were observed across treatments in the time spent as third instar, prepupa and pupa. However, except in the case of pupal development time, there was no difference between the control diet and the diet containing the higher concentration of Sudan Red 7B. Male lacewings had shorter second and third instar development periods that resulted in a significantly shorter overall development time (Table 8.1). Similar minor effects were observed by Qureshi et al. (2004) and Vilarinho et al. (2006) in comparisons across treatments of diets containing Sudan Red 7B to mark *Diatraea grandiosella* Dyar and *Spodoptera frugiperda* (J.E. Smith), respectively. The inconsequential differences observed in *C. carnea*

development time, even at high concentrations, lead us to conclude that the dye does not affect the vigour factor in this species. Male lacewings had shorter second and third instar development periods that resulted in a significantly shorter overall development time (Table 8.1).

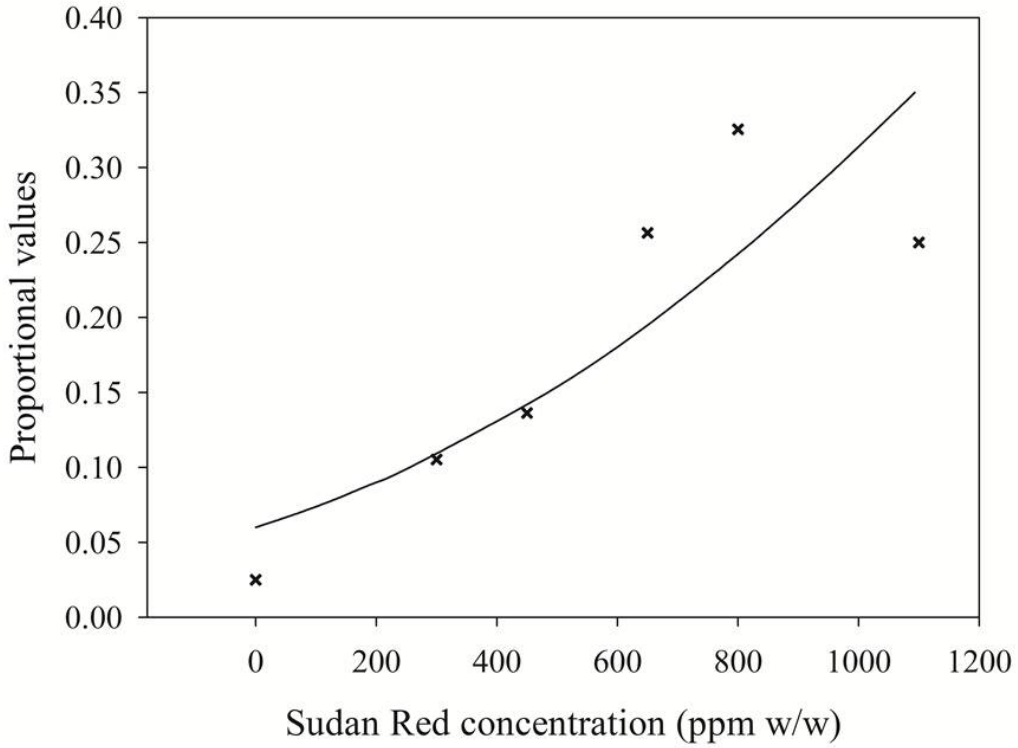

Figure 8.1. Relationship between Sudan Red 7B concentration in the artificial diet and *C. carnea* larval mortality rate calculated using probit analysis (n = 50, slope = 0.0011 ± 0.0003 (mean ± SE), xP^2P = 14.19).

The different red dye concentrations had little effect on the larval weight increase during the development period or on final pupal weight (Table 8.2). From day 6 to 8, weight increase differences were observed between the control and several higher concentration diets (650, 800 and 1100 ppm). Nevertheless, no differences across treatments were observed in the other periods evaluated or in the 12h pupa weight. *C. carnea* showed a similar trend in development time and weight increase, with no substantial differences recorded across diets. In general, females underwent a higher weight increase compared to males, resulting in a higher final 12 h pupae weight (Table 8.2).

Table 8.2. Mean larval and pupal weight increase (± SD) measured at different time intervals of *C. carnea* individuals fed on artificial diet with increasing concentrations of Sudan Red 7B and two-way ANOVA results.

Diet	Sex	*n*	Larval and pupal weight increase (mg)[a]					
			0 - 2 d larva	2 - 4 d larva	4 - 6 d larva	6 - 8 d larva	8 - 10 d larva	12 h pupa
Control	Both	41	0.44 ± 0.11	0.39 ± 0.28	1.43 ± 0.48 ab	2.78 ± 1.59	7.27 ± 2.26	11.71 ± 1.66
Red diet 300 ppm	Both	34	0.44 ± 0.13	0.34 ± 0.17	1.47 ± 0.43 a	2.66 ± 1.19	7.21 ± 1.85	10.95 ± 1.71
Red diet 450 ppm	Both	38	0.38 ± 0.15	0.26 ± 0.19	1.37 ± 0.55 abc	2.02 ± 1.12	6.21 ± 2.73	11.55 ± 2.05
Red diet 650 ppm	Both	29	0.38 ± 0.15	0.30 ± 0.25	1.16 ± 0.52 c	2.42 ± 1.79	6.35 ± 2.41	11.24 ± 2.20
Red diet 800 ppm	Both	31	0.44 ± 0.10	0.39 ± 0.21	1.15 ± 0.47 c	2.60 ± 1.36	6.85 ± 2.32	10.98 ± 1.67
Red diet 1100 ppm	Both	30	0.43 ± 0.12	0.35 ± 0.17	1.23 ± 0.43 bc	2.30 ± 1.33	6.40 ± 2.48	10.48 ± 1.68
All diets	Female	96	0.43 ± 0.14	0.30 ± 0.20 *a*	1.42 ± 0.51 *a*	2.34 ± 1.27	7.51 ± 2.61 *a*	12.48 ± 1.51 *a*
All diets	Male	107	0.41 ± 0.12	0.37 ± 0.23 *b*	1.22 ± 0.46 *b*	2.59 ± 1.54	6.04 ± 1.88 *b*	10.07 ± 1.33 *b*
Diet (df = 5)								
F			1.77	1.97	2.54	1.62	1.44	1.33
P			0.120	0.085	0.030*	0.156	0.212	0.255
Sex (df = 1)								
F			1.45	4.63	6.98	1.27	22.11	125.43
P			0.229	0.044*	0.009*	0.260	< 0.001*	< 0.001*
Diet × Sex (df = 5)								
F			1.40	1.62	1.22	2.88	0.66	0.69
P			0.227	0.330	0.300	0.015*	0.657	0.630

[a] Means (± SD) within the same column, followed by the same or no letter, are not significantly different, Fisher's least significance difference test ($P < 0.05$). Regular letters indicate differences in the factor diet. Italic letters indicate differences in the factor sex.

Table 8.3. Mean larval diet consumption (± SD) measured at different time intervals of *C. carnea* individuals fed on artificial diet with increasing concentrations of Sudan Red 7B and two-way ANOVA results.

Diet	Sex	*n*	Diet consumed per time interval (mg)[a]					
			0 - 2 d	2 - 4 d	4 - 6 d	6 - 8 d	8 - 10 d	Overall
Control	Both	41	3.17 ± 2.73 a	2.10 ± 1.57	12.42 ± 4.95 a	8.76 ± 5.45	25.10 ± 8.57 a	51.54 ± 14.15 ab
Red diet 300 ppm	Both	34	2.17 ± 1.15 bc	2.53 ± 1.56	9.78 ± 3.76 b	7.51 ± 3.67	23.79 ± 8.77 a	45.73 ± 10.91 bc
Red diet 450 ppm	Both	38	1.42 ± 0.80 b	2.89 ± 2.26	7.43 ± 4.01 c	6.85 ± 4.82	22.40 ± 9.57 a	40.64 ± 12.48 c
Red diet 650 ppm	Both	29	1.94 ± 1.43 bc	2.60 ± 1.56	9.59 ± 4.41 b	8.54 ± 6.50	27.60 ± 14.46 b	51.14 ± 10.19 a
Red diet 800 ppm	Both	31	2.59 ± 1.51 ac	2.42 ± 1.70	12.74 ± 6.72 a	6.72 ± 3.03	22.54 ± 7.08 a	46.70 ± 13.44 bc
Red diet 1100 ppm	Both	30	2.40 ± 2.37 c	2.30 ± 2.01	7.94 ± 3.61 bc	7.66 ± 3.96	25.95 ± 9.42 a	47.01 ± 10.07 bc
All diets	Female	96	2.13 ± 1.67	2.20 ± 1.62	10.17 ± 4.60	7.31 ± 5.18	25.95 ± 14.83	47.95 ± 16.72
All diets	Male	107	2.46 ± 2.09	2.71 ± 1.92	9.92 ± 5.48	8.05 ± 4.29	24.35 ± 10.32	47.58 ± 15.30
Diet (df = 5)								
F			3.88	1.02	8.26	1.21	2.55	4.07
P			0.002*	0.405	< 0.001*	0.350	0.029*	0.002*
Sex (df = 1)								
F			1.73	3.20	0.02	0.67	0.99	0.11
P			0.121	0.75	0.967	0.413	0.320	0.739
Diet × Sex (df = 5)								
F			0.72	1.63	1.61	1.80	1.07	1.32
P			0.608	0.155	0.159	0.114	0.379	0.257

[a] Means (± SD) within the same column followed by the same or no letter are not significantly different, Fisher's least significance difference test ($P < 0.05$). Regular letters indicate differences in the factor diet. Italic letters indicate differences in the factor sex.

Significant differences were observed across diets in overall larval food ingestion (Table 8.3). Individuals fed on the control diet consumed more than those fed on the 450 ppm diet. Nevertheless, no difference was observed between the control diet and the diets containing the highest concentrations of Sudan Red 7B (800 and 1100 ppm). *C. carnea* larvae consumed diets with high concentrations of Sudan Red 7B in a normal way, and, despite the different consumption rates across treatments, there does not appear to be any relationship between dye content and diet acceptance. An evaluation of Sudan Red 7B marking diet acceptance has been previously carried out only by Qureshi et al. (2004), who found that *D. grandiosella*'s consumption of the red diet significantly exceeded that of the control diet. They argued that the increased consumption might have been related to the necessity for an extra amount of energy to metabolize the dye. This factor, assumed to be concentration-related, has not been observed in the current study. Males and females had very similar food ingestion rates over the time periods monitored and recorded roughly identical overall consumption rates. Heavier female pupae do not seem to be related to higher rates of diet consumption.

8.4.3 Effects on Adult Fecundity and Survival

Adults fed on diets containing lower concentrations (300 ppm) exhibited slight or no changes in color. On this basis, Sudan Red 7B concentrations below 450 ppm should not be used for marking *C. carnea*. Higher concentrations yielded a readily identifiable reddish coloration of *C. carnea*'s abdomen. The marking was visible enough not to require further examination of the insect under the stereoscope or abdomen dissection. Furthermore, if in doubt, the marking can be revealed by squeezing the abdomen and releasing the dye accumulated in the internal organs. *C. carnea* survivorship differed from the control at 450 and 650 ppm (Fig. 8.2), but no differences were observed between the control and higher concentration values. The differences observed indicate that the dye affects adult survival at low and medium concentrations. As larval mortality increased at high concentrations, surviving individuals might have been able to cope with dye toxicity at the larval and pupal stages, thus attenuating the toxic effect on the adult stage as discussed

by Vilarinho et al (2006) for *S. frugiperda*. No statistical differences were observed between males and females (Cox regression, Z = 0.17, P = 0.873), which had almost identical life spans of 83.8 ± 38.8 (Mean ± SD, n = 75) and 84.5 ± 39.7 days (n = 64), respectively.

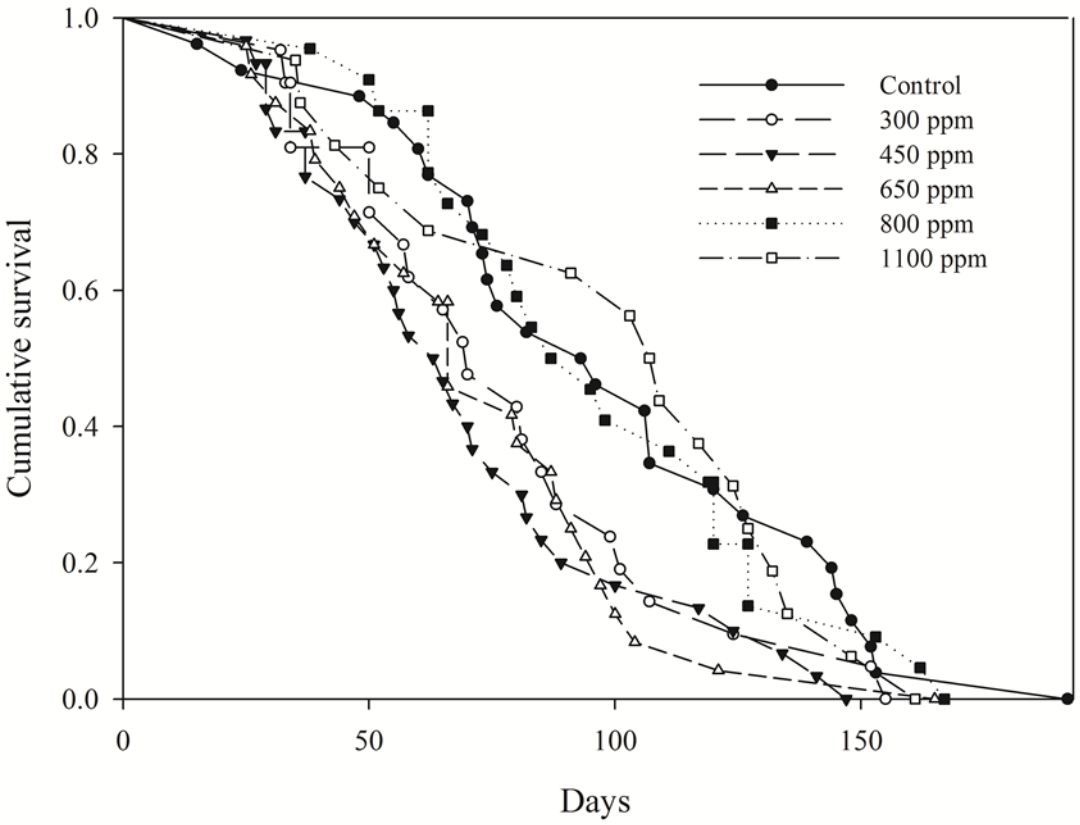

Figure 8.2. Cumulative proportional survival (Kaplan–Meier) curve of *C. carnea* adults emerging from larvae fed on the following diets: control (Cox regression factor baseline, n = 24), 300 ppm (Cox regression, Z = 1.7, P = 0.087, n = 21), 450 ppm (Cox regression, Z = 2.74, P = 0.006, n = 30), 650 ppm (Cox regression, Z = 2.51, P = 0.024, n = 24), 800 ppm (Cox regression, Z = 0.13, P = 0.894, n = 22) and 1100 ppm (Cox regression, Z = 0.12, P = 0.900, n = 16).

Marked females were able to oviposit normally. The highest rates of oviposition were recorded by control individuals (Table 8.4), and, despite the noticeable mean differences, none were significant due to highly variable rates of oviposition among females. The eggs deposited by *C. carnea* marked females presented no visible change in colour independently of the concentration used. Unlike that which happens in several species of lepidopterans which lay white eggs that are easily marked by the dye, the light green coloration of *C. carnea*

eggs possibly hid any remaining dye transferred (Qureshi et al. 2004; Vilarinho et al. 2006). Eggs deposited by marked females produced viable offspring. The reddish-pink abdominal coloration of marked adults remained visible for at least several months, and as a result, most of the adults died even before the marking started to gradually fade away.

Table 8.4. Mean number of eggs (± SD) per female throughout their complete life span of *C. carnea* individuals fed on artificial diet with increasing concentrations of Sudan Red 7B and ANOVA results.

Diet	*n*	No. Eggs
Control	14	118.9 ± 66.3
Red diet 300 ppm	11	80.5 ± 80.3
Red diet 450 ppm	17	51.6 ± 64.0
Red diet 650 ppm	13	84.0 ± 58.0
Red diet 800 ppm	11	56.5 ± 41.0
Red diet 1100 ppm	9	97.7 ± 70.7
(df = 5)		
F		0.79
P		0.563

8.4.4 Effects on Adult Flight Performance

The LC_{15} concentration (484.6 ppm) was chosen to feed larvae for the adult flight performance test, as this concentration yielded appropriately marked adults at high survival rates. Marked adults showed flight duration, distance moved and mean velocity values very similar to control individuals, and thus, statistical testing revealed no significant mean differences (Fig. 8.3). No differences were observed among males (n = 30) and females (n = 34) in terms of flight duration (ANOVA, F = 0.2, df = 1, P = 0.632) and distance moved (ANOVA, F = 0.3, df = 1, P = 0.603). However, females flew significantly faster, at a mean velocity of 41.5 ± 5.4 cm/s (mean ± SD), than males, which flew at 38.7 ± 3.8 cm/s (ANOVA, F = 5.5, df = 1, P = 0.022). Flight durations and distances varied greatly in both treatments and genders, ranging from 2.2 to 289.2 min and from 43.4 to 6492.1 m, repectively. The data distribution of these variables was moderately skewed towards short flights. Ostlie (1984) obtained similar results when testing marked *O. nubilalis* flight performance and observed that the dye had no significant effect. A similar flightmill study

observed that Sudan Red 7b produced a reduction in flight speed for marked *H. armigera* males, although no effect on flight duration and distance covered was recorded (Zhao et al. 2008). As in the case of *C. carnea*, the red dye does not appear to interfere with normal flight behaviour tested under laboratory conditions. However, few studies have undertaken flight experiments to establish the possible negative effects of dye marking which are so important for the effective application of the marking method in field dispersal studies.

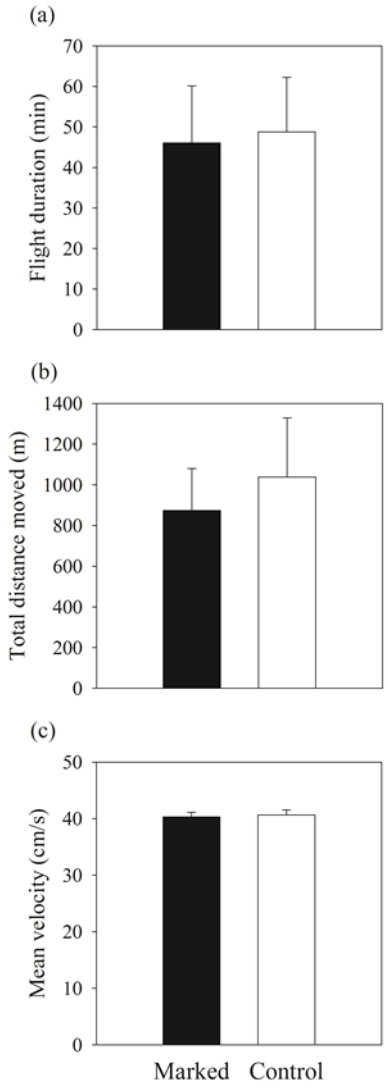

Figure 8.3. Flight performance parameters of *C. carnea* adults reared on Sudan Red 7b at LC_{15} concentration diet (n = 30) and control diet (n = 34). Mean ± SE (a) flight duration (ANOVA, F = 0.3, df = 1, P = 0.597), (b) total distance moved (m) (ANOVA, F = 1.0, df = 1, P = 0.316) and (c) mean velocity (cm/s) (ANOVA, F = 0.1, df = 1, P = 0.700).

In conclusion, according to our results, the Sudan Red 7B dye incorporated into the semisolid artificial diet for lacewings is an effective marker of *C. carnea* adults, since no negative effects were observed for most of the biological parameters evaluated. The range of concentrations tested allowed us to establish an approximate threshold for reliable marking of

adults, as we observed significant variability in colour intensity in the abdomen and other parts of the insect's body. Along with the relationship found between increases in concentrations and mortality, we have managed to obtain concentrations such as LC_{15} and LC_{20} that guarantee both readily identifiable marking and low mortality rates. This information is valuable for optimizing mass rearing procedures with a view to carrying out MRR. Marked adults, despite the life span reductions observed, lived for long periods, possibly long enough to suit the needs of any MRR experiment. The long-lasting, almost permanent, marking allows the dispersal of this chrysopid to be monitored for almost its entire lifespan. Furthermore, since the marking checks are non-destructive, marked individuals can be captured and identified directly in the field and then re-released, which is advantageous if re-sampling of marked *C. carnea* is required over time. This labelling technique could be applicable to other species of similar appearance within the Chrysopidae family. In this respect, in recent experiments, we have successfully marked the species *Dichochrysa prasina* (Burmeister) and obtained adult individuals with clearly discernible pink to reddish segments in the alimentary canal and a pink coloration in the abdomen, sometimes extending to parts of the thorax and head (M.P., unpublished data). Just like *Chrysoperla spp.*, *Dichochrysa spp.*, among which *D. prasina* stands out as a cosmopolitan and abundant species (Pappas et al. 2007), are known to have great potential as biocontrol agents in agroecosystems (Daane 2001). This marking technique may therefore be useful in adult MRR experiments carried out on certain chrysopids.

Acknowledgments

The authors would especially like to thank Rafael Alcalá, María Luisa Fernández Sierra and Herminia Barroso for their laboratory work and assistance and Dr. Francisca Ruano from the University of Granada for her contributions in the early stages of the study. We would also like to thank Michael O'Shea for proof-reading the manuscript. The study was financed by project P07-AGR-02747 (Junta de Andalucía).

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9. General Discussion

Several aspects of entomological research on chrysopids have been addressed in this thesis in relation to their conservation and enhancement in olive orchards. Field studies combined with innovative laboratory methodologies have given insight into some of the effects that traditional, or relatively new, management practices are expected to cause to the species of this family of predators. As the tendency in current agricultural policy and consumer's demands points towards more sustainable agricultural management, the information on agricultural practices impact on assets of economic importance, such as natural enemies, gains increasing relevance Hence, a good deal of the information in this thesis may be directly applied in decision making strategies for biodiversity conservation and conservational biological control in a context of integrated pest management and conservational agriculture in olive cropping. It is noteworthy to mention that methodological advances in several aspects of green lacewings research, such as behavioral studies, the development of a new marking technique, better canopy sampling methods and adjusted sampling effort, may contribute to ease experimental design and widen the range of experimental procedures available to continue increasing the knowledge on these group of species and on other species that may be found of interest in olive orchards. Based on this knowledge, a better management of these insects may eventually help to reduce or even suppress the need for conventional pest control methods.

9.1. The use of automated video tracking technology for behavioral studies of chrysopids

An important part of the present thesis has been devoted to the development of methodological techniques based on video tracking to assess behavioral changes in the test species *C. carnea s.l.* This approach is advantageous compared to other methodologies in many ways (Noldus et al., 2001; Noldus et al., 2002). Apart from its automatic functioning that allows monitorization for long periods of time, its accuracy has been pointed out as one of its most useful characteristics (Delcourt et al., 2006; Mukhina et al., 2001). Thus, in Chapter 3 the movements of *C. carnea s.l.* larvae subjected to different feeding situations elicited different behavioral responses as expected.

The combination of the variables measured explicated successfully these behavioral differences indicating that the tracking had worked properly.

Automated video systems based on digital imaging have been successfully used to track the behavior of other natural enemies such as spiders, predatory mites, coccinelid larvae and parasitoids (Drost et al., 2000; Ortega-Escobar, 2002; Ruzicka and Zemek, 2008). In the case of chrysopid predatory larvae, this is the first time that such methodology has been employed and the results are promising as they open the window for multiple applications of this technology in their research. The EthoVision system was developed to measure mainly three elements: brief behavioral responses within long periods of inaction, behaviors that occur throughout long periods of time, and spatial displacement measurements (Noldus et al., 2001). For the study of lacewings larvae, spatial displacement information is highly relevant. As indicated in Chapter 4, chrysopid larvae moving capacity is essential for their biological control efficiency. Prey encounter and consumption is a function of moving activity as chemical cues are only thought to be influential within short distances (Canard, 2001). Thus, circumstances negatively affecting this movement capacity would be involved in a predation reduction resulting in less beneficial action. Reduction of the moving capacity, for instance, may be among the sublethal effects of pesticides (Desneux et al., 2007) and could greatly affect chrysopids performance, and ultimately, their populations.

In this context, in Chapter 5 the use of automated tracking was particularly useful. Kaolin clay, even though reported to produce direct acute mortality to certain insects pests (Barker et al., 2006; Puterka et al., 2005), was a priori expected to produce only sublethal type of effects due to the lack of insecticidal properties (typical of chemical insecticides). The capacity of the tracking system to identify even subtle changes in translocation parameters was essential to observe effects that otherwise would have been impossible to ascertain. Such little changes are only measurable through highly accurate systems as the one employed. As discussed in Chapter 4, small effects detected in the monitored arena over a short period of time are expected to be translated into significant changes at field scale over the total preimaginal

developmental period. Hence, the video tracking methodology described in Chapter 3 may be applicable as an indirect measure of chrysopid predatory performance in the field since, as exposed above, is directly related to their searching capacity. Apart from the mentioned sublethal effects testing, this methodology may be used to determine factors favoring the fitness of the predator for a higher searching efficiency such as better nutritional quality of the consumed preys.

The option of this video tracking system that allows to mark and define special areas of interest was also rather useful for the behavioral evaluation carried out on *C. carnea s.l.*. In Chapter 3 the "zoom" into the feeding influence area allowed to detect and register through the movement parameters the sinuous twisting and turning movements typical of chrysopid prey handling (Principi and Canard, 1984). In Chapter 4 we used zone definition to test the preference of *C. carnea s.l.* for different surfaces and its movement efficiency on each one of them. Movement parameters, such as mean velocity and distance covered, were used to evaluate the movement capacity, and time spent on each surface was used as an indication of preference.

A slight reduction on movement parameters was detected on treated surfaces, which again at field level, could be related to an important decrease of the locomotory capacity. The experimental design used in this experiment allowed testing the same individual on different surfaces at the same time (recording just one track). Therefore, two sets of behavioral data were obtained per tested individual, one for the kaolin-treated surface, and one for the kaolin free surface. This simultaneous testing procedure was adopted as it was expected to provide better accuracy than testing the treatments sequentially by eliminating the any possible temporal variability in the movement capacity of the tested larvae. As for other applications of this kind of experiments, the same experimental design may be used to establish what plant surface characteristics favor chrysopid searching activity. The use of selected cultivars that allow higher pest control efficiency is among the practices available for conservation biological control (Van Driesche et al., 2008). In this sense, plant surface waxiness and hairiness have been observed to inhibit *C. carnea s.l.*

searching movements (Arzet, 1973; Elsey, 1974). This type of leaf surface suitability evaluations have been previously performed on predatory mites using EthoVision system (Krips et al., 1999).

We also observed a predilection towards kaolin free substrates in comparison to kaolin-treated ones. This preference was established based on small differences in the time spent in each zone. Due to the relatively small size of the arena, *C. carnea s.l.* larvae were forced to spend a certain amount of time on the kaolin-covered surface. As in the case of movement results obtained in Chapter 4, a more marked repellent effect of treated surfaces may be expected in the field. However, results on preference effects measured through this system at laboratory level seem a priori harder to generalize at field level. Larvae may just avoid kaolin-treated surfaces by turning around at the moment the sense any movement impediment or, by contrary, continue moving on these surfaces. Marking zones of influence in EthoVision experimental designs and evaluation the time spent on each zone offers many possibilities for chrysopids behavioral research. Thus, more understanding may be gained on the influence of chemical cues at short distances using the system to determine chemical attraction or repulsion. This kind of experiments, traditionally carried out with olfactometers, have been successfully adapted to the EthoVision system for testing other insects (Ruzicka and Zemek, 2008; Stewart-Jones et al., 2006; Stewart-Jones et al., 2007).

In Chapter 8 the EthoVision systems was used in combination with a flight mill to determine any possible effects of the marking system on *C. carnea s.l.* flying activity. Flight mill parameters may be measured in different ways. The most basic option is to count directly the number of revolutions (Shelton et al., 2006), however, the measurements are usually taken using and infrared emitter and receptor as an activity sensor. A pulse is transmitted directly to a computer every time an infra-red light beam is interrupted by the flight mill arm recording the number revolutions per unit of time (Blackmer et al., 2004; Taylor et al., 2010; Wu et al., 2006). This raw information allows calculating the main flight performance parameters i.e. flight speed, duration, and

distance covered. Adapting the flight mill methodology to the EthoVision involved a change in the way parameters were calculated. Thus, EthoVision tracked the object in motion with precision (the black cardboard situated opposite to the flying *C. carnea s.l.* adult) calculating directly the parameters based in its translocation and not from indirect information as the number of revolutions. Direct measurements improve the accuracy of the resulting parameters establishing with higher precision when the lacewing is in motion and when it ceases flight. This is achieved by preventing simple inertial movements from being registered as actual movement, as it may happen using activity sensor in case the rotation arm triggers a responses when the insect has stopped flying. Although the combination of the EthoVision detection system with the flight mill was highly useful for the purpose of our experiment, the use of video tracking for recording chrysopids flight mill parameters has a very important drawback. Just one test can be carried out at a time due to detection requirements. On the contrary, traditional flight mill systems are comprised by several individual flight mills connected to the same computer allowing testing many insects at the same time. Liu et al. (2011) tested up to 32 individuals of *Chrysoperla sinica* (Tjeder) simultaneously in one of the very few flight performance evaluations that have been carried out in chrysopids.

9.2 Contribution to the evaluation of the effects of pest control methods on chrysopids

C. carnea s.l. has been widely used in pesticides side-effects tests as a representative of the family Chrysopidae and is currently one of the most important test species for regulatory requirements (Vogt et al., 2001). The testing procedures for chemical pesticides is well established by the IOBC working group consisting on a sequential scheme that includes laboratory, semi-field and field methods (Bigler and Waldburger, 1994). Laboratory testing protocols and similar experiments are fully applicable with reliable results to novel pest control compounds expected to work through similar mechanism as insecticides. Products such as Spinosad® (Dow AgroSciences) and insect's growth regulators have been thus tested on *C. carnea s.l.* (Mandour, 2009; Medina et al., 2004; Medina et al., 2003). For new technologies, such as

genetically modified plants novel test methods are starting to be used based on tritrophic feeding experiments (Dutton et al., 2002; Rodrigo-Simón et al., 2006). As in these experiments, the established methodology for insecticide testing was not suitable to establish the side effects of a compound with a completely different mode of action as is the case of kaolin particle films. The preexistent knowledge on its impact on pest insects indicated that a combination of different effects was responsible for the pest suppression achieved by using this pest control method (Puterka et al., 2005; Unruh et al., 2000). Thus, side effects testing on *C. carnea s.l.* demanded a holistic approach with individual, but complementary, experimental strategies looking into the most probable effects of kaolin and including all *C. carnea s.l.* stages.

From the set of experiments carried out on immature stages (the a priori most susceptible) it was concluded that negative effects of the treatment may be a combination of reduced mobility and accidental detachment from the olive plant. The extents to which these effects occur at field level determine whether kaolin treatments cause a relevant negative effect or not, since, as expected, the product caused no acute mortality on immature stages. The negative effects of kaolin film observed on *C. carnea s.l.* may be expected to be found in other predators with similar predatory behavior such as heteropteran predators and spiders. Equally, other pest control strategies with modes of action similar to kaolin (e.g. copper oxychloride and summer oils) may cause similar negative effects to predators. Movement and grasping ability experiments carried out on these other products may help to test this hypothesis.

Not only negative effects of kaolin were found on *C. carnea s.l.* The preference of female adults for kaolin-treated surfaces for oviposition was clearly established. Could this translate into a higher number of larvae present on kaolin-treated trees? In fact no attractive effect of the product was found at field level. However, in such case, this increased presence due to a higher number of eggs would surely compensate for the negative effects observed on larvae.

In Chapter 5 the response of the green lacewing community to the a priori most impacting agricultural practices in olive orchards was assessed in order to test the compatibility of their use with the presence of these natural enemies. The insecticide dimethoate, one of the most widely used pest control methods in olive cropping, affected the Chrysopidae community in general but did not affect the abundance of the dominant species in olive orchards, *C. carnea s.l.*. As discussed in Chapter 5, the notion that chrysopids in general are insecticide resistant insects is refuted by the effect observed in the whole green lacewing community and on *Dichochrysa* spp. particularly. This fact questions the validity of sibling *Chrysoperla* spp. as representative of the Chrysopidae family in insecticide testing for regulatory purposes as, apparently, show a different response compared to the majority of Chrysopidae species present in olive orchards. *C. carnea s.l.* is in general the most numerous chrysopid in agricultural ecosystems (Duelli, 2001), and therefore, despite this fact, and from a conservation biological control point of view, its use for pest control management strategies assessment is unquestionable. However, data on tests carried out on *Chrysoperla* spp. should be extrapolated with caution to field situations where other Chrysopidae species, especially belonging to other genera, are suspected to play an important role in biological control of pest insects.

9.3. The influence of vegetation cover on chrysopids presence

The studies carried out on the chrysopids present in olive orchards under different management regimes during the years 1999, 2000 (Chapter 6) revealed an important relationship between the lack of use of herbicide and *C. carnea s.l.* adults presence on tree canopies. Even though a negative effect was observe on *Chrysoperla* larvae, *Dichochrysa* larvae were very numerous in orchards where no weed suppression had been carried out in the years 1999, 2000 and 2003. This was considered an indicative of a possible positive effect of vegetation cover on *Dichochrysa* spp. adults that could not be tested due to the low amount of individuals collected. Based on this information, in Chapter 7, the effect of vegetation cover was investigated separately from other

management factors by using a large but homogeneous olive orchard. The experimental design also tried to reduce the influence of environmental factors that affect green lacewings populations locally and, additionally, different sampling methodologies were employed aiming at collecting higher number of individuals. Thus, both years of the experiment the amount of *C. carnea s.l.* and *Dichochrysa prasina* (Burmeister) adults recorded in vegetation cover plots were higher than those collected in control plots which confirmed in part the results obtained on Chapter 6. However, unlike in the data from years 1999 and 2000 (Chapter 6), higher *C. carnea s.l.* adult abundance resulted in higher larval presence in trees. As discussed in Chapter 6, biotic factors affecting *C. carnea s.l.* larval populations at local level might explain these differences obtained on the effect of vegetation covers between Chapter 6 and Chapter 7. Ants and several parasitoids have been described as antagonists of chrysopid eggs and larvae in olive orchards (Campos, 2001; Morris et al., 1998). These factors could not possibly affect the results obtained from the experiment described in Chapter 6 as both treatments (vegetation cover and bare soil control) were under highly similar environmental conditions. Concerning *Dichochrysa* spp. larvae, no effect of vegetation cover was recorded in Chapter 7 whilst an important positive relationship was observed in Chapter 6.

In Chapter 7, more *Dichochrysa* spp. adults were present in vegetation cover plots but this did not result in a higher abundance of larvae on tree canopies. The inconsistent results may be due to several factors. Firstly, it must be taken into account that orchards with no managed vegetation cover, sampled in Chapter 6, had generally received insecticidal treatments that, in the long term, had certainly reduced *Dichochrysa* spp populations substantially. Secondly, in Chapter 6 the analysis was extended to the complete period of green lacewings activity as the weed control was carried out throughout the whole year. Unlike in Chapter 6, in Chapter 7 the vegetation cover elimination was carried out only before summer as the study was focused on the increase of chrysopid populations with a potential incidence over the carpopahgous generation of *Pray oleae* (Bernard). *Dichochrysa* larvae presence on trees was delayed with respect to *Chrysoperla* larvae, and therefore, despite vegetation cover attracted more adults, their peak of oviposition on trees took place when the attractive effect of vegetation cover

had already disappeared due to the senescence occurring until June. In organic and integrated orchards sampled in Chapter 6, the proliferation of late-flowering weedy plants occurring from the month July could have benefit *Dichochrysa* oviposition on trees compared to bare soil conventional orchards in 1999, 2000 and 2003.

There are several more results from the field experiments that suggest the importance of late flowering weeds on chrysopid presence after the spring vegetation cover has disappeared at the beginning of the summer. In the integrated orchard sampled in 1999 and 2000 (Chapter 6), the suppression on the second simazine treatment in the year 2000 (usually applied in early autumn) resulted in a spectacular increase in *C. carnea s.l.* adults in September and October. In Chapter 7, in September 2000 a higher abundance of *C. carnea s.l.* adults was observed in former control plots compared to vegetation cover plots (Chapter 7, Fig. 7.5). At this time, no difference was to be expected as the spring vegetation cover had already disappeared and later weeds would develop in the same conditions in both treatments. However, field observations carried out in September revealed that more late-flowering weeds were present in the former control plots compared to plots where vegetation cover had senesced. The vegetation cover mulch remains prevented new weedy plants from developing in between olive tree rows (Porcel, personal observation). Hence, common plants such as *Dittrichia viscosa* (L.), traditionally linked to olive cropping and that flower from September to October (Franco-Micán et al., 2008), may possibly help to increase and conserve green lacewings populations locally acting as an feeding source in moments of food scarcity in the field.

The results obtained from the fieldwork experiments established a clear relationship between the presence of a plant and floral diversity within olive orchards and the abundance of the main chrysopid species present in this agricultural ecosystem. Nevertheless, the study of the larvae present on olive trees revealed that the increased presence of adults does not necessarily translate into more larvae available for biological control of pests on the tree canopies. In Chapter 7 we hypothesized that *C. carnea s.l.* are able to move

from vegetation cover to the tree based on data collected with different sampling methods. Establishing the occurrence of these changes of substrate is essential to determine whether the provision of flowering plants may be considered or not for use in conservation biological control with chrysopids. The methodology for lacewings marking developed in Chapter 8 may be highly useful for adult movement studies with biological control applications. Thus, short scale movement of chrysopids from provided resources to crop plants, such as the one described above, may be confirmed and detailed. Furthermore, this methodology may also help to bring an insight into the role of natural vegetation patches, believed to be important recolonization sources (Szentkirályi, 2001a), and therefore, on the relevance of this agricultural landscape feature in conservation biological control related to chrysopids.

9.4. Chrysopid species assemblages and seasonality

It was concluded from the results obtained in 1999, 2000 and 2003 (Chapter 6) that management factors have a significant influence on chrysopid species assemblages expected to be found in southern Andalusian olive orchards. Conventional orchards can be generalized to present a marked dominance of *C. carnea s.l.* while a higher diversity may be probably registered in organically managed orchards and those subjected to integrated production regulation. Particularly, and according to these results, more species of the genus *Dichochrysa*, the most numerous genus in number of species in olive orchards (Szentkirályi, 2001b), may be expected to occur under more sustainable regimes. These results are highly consistent with those obtained later, in the years 2009 and 2010 (Chapter 7). The orchard used these years had been under integrated production for long and, even though pesticides are allowed under certain circumstances, their use had been fairly rare. In addition, vegetation cover had been managed for more than 10 years in the total extension of the orchard contributing without doubt to a long-term establishment of a rich chrysopid community. Consequently, a high diversity of chrysopids was recorded in this orchard. Thirteen different species were collected during the two sampling periods, of which 6 belonged to the genus *Dichochrysa*. Additionally, as in the organic and some other integrated

orchards (Chapter 6) an important amount of *Dichochrysa* larvae were collected from olive trees.

Seasonality of species occurrence was also highly coincidental in the field studies (Chapter 6 and 7). On tree canopies (suction and beating samples), both adult and larvae of *C. carnea s.l.* were by far the most abundant during the spring and early summer. *Dichochrysa* spp. larvae started to appear on olive canopies later, peaking in the month of August (Chapter 6, Fig. 6.2 and Chapter 7 data not presented) long after spring vegetation cover had disappeared. The results obtained confirm, in opposition to the observations made by Neuenschwander and Michelakis (1980) and Alrouechdi et al. (1980), that at least in Andalusian olive orchards *Dichochrysa* spp. usually develop using the olive tree as host plant and may be roughly around a 20% of the total larvae throughout the whole year. A similar conclusion was reported by Pantaleoni et al. (2001) based on eggs collection from a Sardinian olive trees. It is noteworthy the scarcity of *Dichochrysa* spp. adults in samples collected directly from the olive tree in comparison with their abundance in McPhail captures during the spring months (Chapter 7). It seems reasonable to think that these species do not rest on the tree during daylight hours looking for refuge on vegetation cover plants or the olive tree trunks. For instance *D. prasina* is believed to show preference for low strata such as herbaceous plants and shrubs (Szentkirályi, 2001a). This preference would also explain the lack of *Dichochrysa* spp. adults registered on organic and integrated orchards where their larvae were found to be relatively abundant (Chapter 6). According to this observation, the attractive effect exerted by vegetation covers may be explained in terms of shelter provision in addition to the effect of food resources provision.

As discussed in Chapter 7, *Dichochrysa* larvae predation upon *P. oleae* eggs should not be discarded despite the fact that *C. carnea s.l.* larvae and adults are highly predominant. *Dichochrysa* larvae are believed to prey mainly on *Saissetia oleae* nymphal stages (Szentkirályi, 2001b). However, the extremely low incidence of this pest in the orchard sampled in Chapter 7 (data not presented), in addition to the presence of *Dichochrysa* adults and larvae

recorded, contradict this association indicating that these species are able to develop optimally on other preys within olive orchards.

The occurrence of other less abundant species also coincided in the experiments carried out in Chapter 6 and Chapter 7. *Rexa lordina* Navás adults and larvae were captured from late April to early June confirming the monovoltinims of this rare species (Canard and Labrique, 1989; Monserrat, 2008). Canard and Labrique (1989) found this chrysopid species associated to the oleaceous plant *Phillyrea angustifolia* L. bearing nymphs of *Euphyllura olivina* Costa. Nymphs of this secondary pest species were present on olive trees of the orchard used in Chapter 7 from late April to late May (data not presented) fully coinciding with the onset of *R. lordina* adults presence detected through McPhail trapping. Hence, it is very possible that the fact that *R. lordina* is exclusively associated to Oleaceae plant substrates may be explained in terms of a strict prey specificity to the olive psyllid. Furthermore, no effect of vegetation cover was observed on *R. lordina* suggesting that adults are not attracted to supplementary food resources as other chrysopid species are (Chapter 7). This could be an indication of the use of olive pollen, abundantly produced in the month of May, as their main food source implying that the olive tree might cover their complete ecological requirements.

Chrysopa spp. seasonality also coincided in the different field experiments carried out (Chapter 6 and 7) appearing in summer and early autumn. It has been appointed the fact that, in McPhail trapping, predatory chrysopid adults may be underrepresented since the bait used to capture lacewings is a priori more attractive for palyno-glycophagous species (Szentkirályi, 2001b). Surprisingly, comparing the results obtained in beating and McPhail trapping samples, the percentage of *Chrysopa* spp. adults is highly similar (Chapter 7, Table 7.1). These results indicate thereby that McPhail trapping is a suitable method in order to establish relative abundance of predatory chrysopid adults.

In the present thesis the species of the *carnea*-group, with the exception of the few individuals of *Chrysoperla meditteranea* (Hölzel) collected in 2010, have been lumped under the taxonomic category of C. *carnea s.l.* Despite the existence of keys to identify these sibling species locally (Villenave, 2007), the high variability of morphological features of southern European sibling species makes very difficult to identify all of them reliably without testing their courtship songs (Henry et al., 2003). Additionally, the species *Chrysoperla lucasina* (Lacroix) characterized by a dark pleural stripe on the first two abdominal segments (Canard and Thierry, 2007; Henry et al., 1996), was not possible to identify due to the sampling methods used and the long term conservation of the specimens that produced, in most occasions, a darkening of the whole individual making impossible to observe this subtle color mark. However, as mentioned in the introductory section of this thesis, not many species of the *carnea*-group are expected to be abundant in olive orchards. *C.c.*4 or *Chrysoperla carnea* (Stephens) *s. str.* (*sensu* Henry) is a central European species not known to be present in warm Mediterranean regions (Henry, 2001; Henry et al., 2002). *C.c.*2 or *Chrysoperla affinis* Henry, Brooks, Duelli & Johnson is believed not to be related to agricultural ecosystems (Henry et al., 2002). *C. lucasina* and *C.c.*3 (*Chrysoperla agilis* Henry, Brooks, Duelli & Johnson) are sympatric species generally found in agricultural sites and present in southern Spain (Henry, 2001; Henry et al., 2003). However, *C. agilis* is better adapted to warmer and drier zones of southern Europe and shows a high dispersion capacity associated to crop specialist chrysopids which has led to believe that it may be an effective biological control agent in places like southern Spain (Henry et al., 2003). Hence, *C. agilis* is the most likely species of the *carnea*-group to colonize Andalusian olive trees and therefore probably the most abundant *Chrysoperla* sp. captured in Chapters 6 and 7.

9.5. References

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10. Conclusions/Conclusiones

From the analysis of the results obtained in the present thesis the following conclusions have been drawn:

1. The behavioral research methodology consisting in the use of the computerized video tracking system EthoVision® to record movements on a 4.5 cm Petri dish arena, illuminated from the underside by using a DCR III fiber optic illuminator, is a suitable methodology for *Chrysoperla carnea s.l* .behavioral studies as it detects correctly, through different movement parameters, changes in behavior related to the presence and the absence of prey.

2. Kaolin suspension direct spray does not affect third instar *C. carnea s.l.* larvae normal development to adulthood or the hatchability of recently laid eggs; however, this treatment reduces third instar larvae movement capacity.

3. Third instar *C. carnea s.l.* larvae show decreased mobility on kaolin-treated surfaces as well as difficulty grasping treated leaves.

4. *C. carnea s.l.* female adults clearly prefer kaolin-treated surfaces for oviposition.

5. The use of the insecticide dimethoate in olive orchards does not affect the abundance of larvae and adults of the species *C. carnea s.l.* However, this insecticide is related to an important decrease, or even the total disappearance, of *Dichochrysa* larvae.

6. Agricultural management intensification in olive orchards is related to a loss in chrysopids biodiversity and to an increase in the dominance of the species *C. carnea s.l.*

7. The presence of resident vegetation cover in olive orchards from early spring, until naturally senescing in June, contributes to increase the

total abundance of adult chrysopids, specifically the species *C. carnea s.l.* and *Dichochrysa prasina* (Burmeister), and chrysopid diversity during this period.

8. The higher number of adults, related to the presence of vegetation cover, results in a higher number of *C. carnea s.l.* larvae on olive canopies. This is not the case of *Dichochrysa* spp. larvae.

9. Feeding *C. carnea s.l.* larvae with an artificial meridic diet incorporating the oil-soluble dye Sudan Red 7B was an effective method to mark internally adults of this species. The dyes Solvent Blue 35 and Sudan Black B are not adequate for this purpose.

10. A 484.6 ppm concentration of Sudan Red 7B in the meridic diet ensures both an adequate marking level and an acceptable larval survival rate with no substantial effects on larval development and diet consumption, or adult fecundity and flight performance.

A partir del análisis de los resultados obtenidos en la presente tesis doctoral se ha llegado a las siguientes conclusiones:

1. La metodología para el estudio del comportamiento basada en el uso del sistema computerizado de seguimiento automático EthoVision® para registrar los movimientos en un placa Petri de 4.5 cm de diámetro como arena, iluminada desde la parte inferior con un iluminador de fibra óptica DCR III, es adecuada para estudios de comportamiento de *Chrysoperla carnea s.l.* ya que detecta correctamente, mediante distintos parámetros de movimiento, cambios en el comportamiento según la presencia o ausencia de presa.

2. La pulverización directa de una suspensión de caolín no afecta a larvas de tercera edad de *C. carnea s.l.* en su desarrollo normal hasta el estado adulto ni a la eclosión de huevos recién depuestos aunque, este mismo tratamiento, reduce la capacidad de movimiento de dichas larvas.

3. Las superficies tratadas con una suspensión de caolín afectan negativamente a la capacidad de movimiento de larvas de tercera edad de *C. carnea s.l.* y dificulta su adherencia a hojas de olivo.

4. Las hembras adultas de *C. carnea s.l.* muestran una clara preferencia por superficies tratadas con caolín para ovipositar.

5. La utilización del insecticida dimetoato en el olivar no afecta a la abundancia de larvas y adultos de la especie *C. carnea s.l.* Sin embargo, este mismo insecticida produce un importante descenso, o incluso la total ausencia, de larvas del género *Dichochrysa*.

6. La intensificación agraria en el olivar está relacionada con una pérdida de la diversidad de crisópidos y un aumento de la dominancia de la especie *C. carnea s.l.*

7. La presencia de una cubierta vegetal espontánea en el olivar desde el comienzo de la primavera hasta su secado natural en junio, contribuye a incrementar la abundancia total de crisópidos adultos, específicamente

de las especies *C. carnea s.l.* and *Dichochrysa prasina* (Burmeister), así como a aumentar ligeramente su diversidad durante este periodo.

8. El mayor número de adultos de la especie *C. carnea s.l.*, debido a la presencia de la cubierta vegetal, se traduce en un mayor número de larvas de esta especie en la copa del olivo. En el caso de larvas de *Dichochrysa* spp no se observa dicho efecto.

9. El tinte Sudan Red 7B incorporado a una dieta merídica para alimentar a larvas de *C. carnea s.l.* es un modo eficaz para marcar los adultos de esta especie. Los tintes Solvent Blue 35 y Sudan Black B no son adecuados para conseguir dicho marcaje.

10. La concentración de 484,6 ppm de Sudan Red 7B en la dieta merídica garantiza un marcaje adecuado y una aceptable supervivencia de larvas, sin causar ningún efecto sustancial sobre el desarrollo larval y su ingesta de alimento, ni sobre la fecundidad y capacidad de vuelo de adultos.

Appendix I. Published article

Biological Control 59 (2011) 98–105

Contents lists available at ScienceDirect

Biological Control

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

Biological and behavioral effects of kaolin particle film on larvae and adults of *Chrysoperla carnea* (Neuroptera: Chrysopidae)

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ARTICLE INFO

Article history:
Received 3 August 2010
Accepted 19 July 2011
Available online 23 July 2011

Keywords:
EthoVision
Green lacewing
Natural enemies
Olive
Predator
Video tracking

ABSTRACT

Several laboratory and field experiments were conducted to investigate the effect of kaolin particle film formulation Surround WP on the biology and behavior of the common generalist predator *Chrysoperla carnea* (Stephens). Kaolin 5% (w/v) suspension direct spray did not affect third instar larvae development to adulthood. The hatchability of recently laid eggs, subjected to the same spraying process, was also unaltered. However, third instar larvae coated with particle film after kaolin spraying showed slightly hampered movement capacity after measuring: distance moved, mean velocity, angular velocity and time spent in motion, obtained using the computerized system EthoVision. Parameters extracted from recorded larvae movement on a kaolin film surface showed similar decreased mobility results as well as preference for the clean control surface. Additionally, the larvae had difficulty grasping treated leaves. *C. carnea* adult females showed a predominant preference for treated leaves in oviposition choice tests. In the field trial, no difference in *C. carnea* adult abundance was found between kaolin-treated and control olive trees. These results indicate that disruption of movement capacity and dislodgement from the plant surface may be the principal negative effects of particle film on *C. carnea* larvae. Despite the positive trend in oviposition towards kaolin treated surfaces, a particle film attraction effect on adults was not observed at field level.

1. Introduction

Kaolin particle technology is a relatively new and promising option for the reduction of pest and disease damage in certain crops. The plants are dusted or sprayed with particles of this non-abrasive, chemically inert, aluminosilicate [$Al_4SI_4(OH)_{18}$] mineral, creating a film that coats the plant and acts as a protective barrier against both pathogens and pest arthropods (Glenn et al., 1999). The use of kaolin as a pest management strategy has proved effective as a broad spectrum compound against a wide range of pest insects such as the psyllid *Cacopsylla pyricola* Foerster on pear, *Diaphorina citri* Kuwayama on citrus (Hall et al., 2007; Puterka et al., 2005), the codling moth *Cydia pomonella* (L.) on pear (Unruh et al., 2000), Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) on apple (Mazor and Erez, 2004), the thrip *Thrips tabaci* Lindeman on onions (Larentzaki et al., 2008) and the aphid *Myzus persicae* (Sulzer) on peach (Karagounis et al., 2006). Specifically, in olive farming, recent research has shown particle film effectiveness in suppressing the olive fruit fly *Bactrocera oleae* Gmel., the key pest in this crop (Pascual et al., 2010; Saour and Makee, 2004) and the black scale *Saissetia oleae* (Olivier) (Pascual et al., 2010). These observations point to the feasibility of kaolin particle films as a viable alternative to extensively using insecticidal control. Particle film technology also has the advantage of being permitted in organic agriculture, which, alongside mass-trapping (Porcel et al., 2009) and naturally derived pesticides (Iannotta et al., 2007), is one of the few options available to organic olive growers to control *B. oleae* damage.

Nevertheless, pest control methods that inhibit the action of harmful insects need to be evaluated for their effects on beneficial arthropods in order to increase our knowledge of the possible impact caused by their application. This knowledge is useful in joint strategies where alternative pest control methods should not interfere with biological control. Recent literature has thus generally focused on field assessment of kaolin effects based on the presence or absence of beneficial arthropods on kaolin-treated crops (Karagounis et al., 2006; Marko et al., 2008; Pascual et al., 2010; Sackett et al., 2007). The mechanisms underlying how particle films affect the biology and behavior of insect pests have been extensively explored by several authors (Cadogan and Scharbach, 2005; Lemoyne et al., 2008; Puterka et al., 2005), who describe a variety of effects: direct toxicity and interference with insects' ability to settle, move or oviposit (Hall et al., 2007). However, to the best of our knowledge, the specific effects of kaolin films on beneficial insects have been the subject of limited study, and in the case of predators,

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doi:10.1016/j.biocontrol.2011.07.011

no information on possible behavioral and biological disruptions is available.

The green lacewing *Chrysoperla carnea s. lat*. (Stephens) (Neuroptera: Chrysopidae) is one of the most common, naturally occurring, arthropod predators (Duelli, 2001). Its extensive range of prey, including almost all soft-bodied arthropods, its wide distribution, as well as its voracious feeding capacity, make this natural enemy a promising candidate for pest management programs (Tauber et al., 2000). Due to its overall importance as a foliage-dwelling entomophagous predator, this insect has been regarded as an appropriate test species for the assessment of novel pest management compounds (Mandour, 2009; Medina et al., 2003). In olive orchards, the green lacewing is considered the major oophagous predator of the olive moth *Prays oleae* Bernard, helping to reduce the economic impact of this pest. It is also known to prey upon less harmful insects such as the black scale and the olive psylla *Euphyllura olivina* Costa (Campos, 2001). Studies undertaken on different crops have detected decreased abundance and alteration of the assemblages of polyphagous predators associated with kaolin treatments (Marko et al., 2008; Pascual et al., 2010; Sackett et al., 2007). Therefore, the aim of the present study is to evaluate both the biological and behavioral effects of kaolin particle films on *C. carnea* predator.

2. Materials and methods

The kaolin clay-based particle film used in the present study was the hydrophilic Surround WP crop protectant manufactured by NovaSource (Salt Lake City, UT, USA). The compound is based on processed hydrous kaolin particles (1.0 ± 0.5 μm ø), with an incorporated synthetic hydrocarbon spreader-sticker that enhances particle adhesion to the plant. The compound, hereafter referred to as kaolin, was applied at a rate of 5% in mineral water (50 g/L) as recommended by the manufacturer. Plain mineral water was used as control, and both were applied in all laboratory experiments using an airbrush spray gun (Model 350, Badger Air-Brush Co., Franklin Park, IL, USA) connected to an air compressor generating a cone spray pattern. The spray gun was fixed by means of an adjustable height laboratory arm at 33 cm over the table surface, preventing excessive air flow over the spraying spot position. The spray gun bottle was constantly in contact with a magnetic stirrer held by the same arm in order to avoid kaolin particle sedimentation during the spraying process.

In all laboratory experiments, with the exception of the experiment described in Section 2.3, surfaces and the larvae themselves were sprayed for a 30 s period. To quantitate kaolin particle retention, preweighed Teflon slides (97 cm^2) were similarly sprayed, and, after a 4 h drying process at 25 °C, were weighed again using an electronic analytical balance (AS 220/C/2, RADWAG Balances and Scales, Radom, Poland). The difference between final and initial weights enabled us to calculate the weight of the kaolin film deposited on each slide. The process was replicated up to 20 times. Finally, mean kaolin film weight was divided by the total surface to obtain the deposit per surface unit (μg/cm^2).

2.1. Rearing C. carnea in the laboratory

The larvae used in all experiments were obtained from *C. carnea* eggs collected from a stock colony established in 2005 with larvae supplied by Koppert Spain (La Mojonera, Almería) and were renewed monthly. Larvae were individually reared in Petri dishes and fed on eggs of *Ephestia kuehniella* Zeller (Lepidoptera, Pyralidae). Adults were kept in boxes with an ovipositing surface and provided with an artificial diet (50% honey and 50% pollen) and mineral water. Both were maintained in a controlled environment cabinet at 25 ± 1 °C, 50–60% RH and a photoperiod of 16:8 (L:D) h.

2.2. Acute mortality to larvae

C. carnea larvae were directly sprayed with the 5% kaolin suspension in order to assess any possible effects on mortality**.** A sufficient number of newly laid eggs were removed from the adult-rearing boxes and developed in the same culture conditions as described above. Newly molted third instar larvae were chosen for the experiment given that previous observations had revealed that molting caused effective removal of the particle layer deposited on the larva's dorsal cuticle when spraying first and second instars. The fact that third instar larvae necessarily undergo both cocoon spinning and metamorphosis with a kaolin film attached makes them a priori more susceptible. The inhibition of these developmental processes, whatever its origin, leads in most cases to the individuals' decease (Cadogan and Scharbach, 2005; Liu and Chen, 2001; Vogt et al., 2001). Recently molted larvae (less than 12 h), homogeneous in age and size, were selected and set aside from the stock. Individuals were transferred by gently tapping the underside of the Petri dish or, when necessary, by using a camel paintbrush, to 4.5 cm ø Petri dishes containing a double filter paper layer in order to prevent the formation of large droplets during the spraying process. Larvae were chilled for 15 min, after which they were sprayed with either kaolin suspension or water. After spraying, the thoroughly wetted larvae typically presented the formation of one or more droplets on the insects' dorsum. The Petri dish was then covered, and the individuals were subjected to a 1 h drying period, after which, in the case of kaolin-sprayed larvae, the particle film became visually apparent. Finally, the filter paper was removed, and the larvae were fed and kept in culture conditions. The experiment used a randomized complete block design (RCBD), with replications consisting of 10 insects per treatment replicated five times over time. Tested individuals were checked daily for survival up to adult emergence and the developmental stage in which mortality occurred was recorded.

2.3. Acute mortality to eggs

Kaolin film applications were tested on *C. carnea* eggs to determine their effects on egg viability and larval hatching suppression. Two bands (17 × 9 cm) of self-adhesive green velvety paper (Sadipal Stationery Papers, Girona, Spain), used as ovipositing surfaces in the *C. carnea* stock colony, were attached upside-down to the rectangular removable lid (36 × 22 cm) of the adults' rearing box containing approximately 100 mixed male and female adults. After 12 h, 30 eggs were selected on each band by discarding the rest. One band was sprayed with the kaolin suspension and the other with the control water. Both treatments were delivered by hand-holding the airbrush at an approximate distance of 10 cm and by spraying the whole band area for 30 s. The eggs were observed under the microscope (mag) to detect small droplets of kaolin suspension. After drying, the suspension droplets produced fragments of particle film adhering to the egg's surface. The lid containing the two treated sets of eggs was kept in a cabinet under culture conditions. After three days, *E. kuehniella* eggs were sprinkled over the bands in order to feed neonate larvae to prevent intraspecific egg predation. From the fourth day onwards, newly hatched *C. carnea* larvae were removed daily from the bands by means of a camel paintbrush, and the eggs were checked daily for 8 d to ensure that hatching had ceased. Data regarding viable and non-viable eggs was recorded under a microscope. The non-viable eggs were identified as those that desiccated as well as neonate larvae that failed to free themselves from the eggshell and

died in the process. The experimental design used was a RCBD, with up to 10 replications being carried out over time.

2.4. Effect on leaf-grasping ability

Larvae's capacity to grasp leaf surfaces covered by kaolin particle film as compared to untreated leaves was assessed. Olive leaves (cv. Picual) were collected, cut, and attached side by side to a 100 cm^2 square glass, forming a continuous leaf surface platform. Several platforms were created as described, using either the upper or lower side of the leaves for each platform. Leaf-covered platforms were pressed by means of a weight and kept at cold temperatures to avoid moisture loss. Following standard spraying methodology, the platforms were firstly sprayed with water and left to dry for 2 h. Newly molted (less than 12 h) third instar individuals were raised and chosen as described in Section 2.2. The experimental methodology adopted to assess differential grasping ability is similar to that described for psyllids by Puterka et al. (2005). Leaf platforms were horizontally mounted on a laboratory arm that enabled them to rotate 180°. The larvae were transferred to the leaf surface and allowed to move for several seconds. When the larva was located in the central part of a leaf (sufficiently away from the edge) and still in motion, the platform was inverted for 30 s to determine whether the larvae could continue gripping the surface or falls off the platform. This procedure was repeated using 10 different larvae, after which the platform was sprayed with kaolin, left to dry and used to assay 10 new larvae on the particle film. The whole process was repeated using the underside of the leaf surface. The experiment was carried out using RCBD and was replicated five times over time. Two new leaf platforms (upper and lower surfaces) were constructed per replication.

2.5. Effect of particle film covering C. carnea larvae on mobility and behavior

Directly sprayed kaolin-covered larvae were assessed for changes in behavioral and locomotor parameters. The same size and age of third instar larvae were selected as in previous experiments, which were sprayed with either kaolin or mineral water. Once sprayed, the larvae were starved for a period of 24 h prior to bioassay. Each individual was moved to a windowless controlled room (22 ± 2 °C, 35 ± 10% RH and 130 lux light intensity), placed in a 4.5 cm Petri dish and viewed with a Panasonic CCTV video camera (Mastsushita Electric Industrial Co., Ltd., Japan). Larval movement was recorded for a period of 15 min, and the track was transferred to a computer as part of the EthoVision XT integrated video tracking system (Noldus Information Technology, Wageningen, The Netherlands). The EthoVision software automatically determines the location of the individual larva in the area and calculates several movement parameters derived from changes in position. The parameters chosen were (1) total distance moved (cm), (2) mean velocity (mm/s), (3) mean angular velocity (degrees/cm) and (4) movement (%). The movement variable was defined as the fraction of time the larvae spent in motion. Parameter descriptions are given in Noldus et al. (2002), and algorithms and calculations are described by Noldus Information Technology (2007). One individual at a time was tested, and each larva was used only once. The experiment was conducted on the basis of a completely randomized design (CRD) over five consecutive days, running 10 to 15 trials of both treatments every day up to a total number of 30 valid replicates per treatment (60 replicates).

2.6. Effect of particle film surface on larval mobility and selection

In this experiment, we determined whether kaolin-covered substrates interfered with larval mobility parameters and whether they showed a substrate preference between treated and control surfaces. As in previous experiments, newly molted third instars were obtained from eggs selected for this purpose. The larvae were subjected to a 24 h starvation period before bioassay. The 4.5 cm Petri dish experimental arena was divided into two surface halves of equal size. Following the usual methodology, one half was sprayed with kaolin and the other half with the control water. A semicircular waterproof plastic was used to cover treatment areas during the spraying process, ensuring that they never came in contact with the particle film, as the treatment was always delivered after the control area had been sprayed and dried for 2 h. Given that only the lower part of the Petri dish was sprayed, the experimental design was restricted to this area by coating the dish's circular edge with Fluon (AGC Chemicals America Inc., Moorestown, NJ, USA), thus preventing the larva from climbing up to the lid. The experimental setup and procedures were similar to those described in Section 2.5. The parameters extracted from the recorded tracks were (1) total distance moved (cm), (2) mean velocity (mm/s), (3) mean angular velocity (degrees/cm), (4) movement (%) and (5) time spent in each zone (kaolin and control). The parameters were calculated for each zone individually for the purpose of comparison. Unlike the previous experiment, each individual trial generated the complete set of variables for both treatments. The experiment was carried out over five consecutive days for a total of 50 replicates. Each block of replicates consisted of 10 trials conducted on the same day with the same experimental arena, and a new arena was therefore sprayed each day.

2.7. Adult oviposition preference

C. carnea adults were placed in an oviposition arena and given the choice between kaolin film and control surfaces. The arena consisted of an 8.5 cm ø × 2.5 cm high Petri dish with two ovipositing leaf surfaces, one upside down attached to the lid and the other attached to the bottom of the Petri dish. The 8.5 cm ø surfaces were made as described in Section 2.4. Upper and lower surfaces were prepared per arena. Before mounting the surfaces on the arena, both were divided into equal semicircular zones and sprayed with kaolin and water, proceeding, as described in Section 2.6, by means of a larger plastic cover. As a result, both leaf surfaces contained particle film and control zones of equal area. The upper surface adhered to the bottom of the dish, and the lower surface to the dish's lid. The lid was placed on the dish so that the kaolin film zones always faced the control zones. Newly emerged (after less than 12 h) adults were coupled and transferred to small rearing boxes (0.9 L), kept in culture environmental conditions and provided with adult food and water. After seven days, individual couples were transferred to the ovipositing arena to lay eggs for a period of 48 h. Water, supplied by a moistened piece of sponge, and food, supplied directly, were attached to the sides of the dish, while avoiding interference with the surfaces. The eggs deposited in each designated area (upper and lower surfaces, kaolin film and control zones) were recorded. Females that did not oviposit were excluded from the experiment. Ten couples were successively assayed in the same arena, and the process was repeated with a total of five different arenas (50 individual replicates). The experiment used a RCBD design, where each arena constituted a different block.

2.8. Effect on adult abundance. Field case study

A field experiment was conducted to determine whether a particle film sprayed on olive trees had an immediate effect on adult lacewing presence. The experiment was conducted in a 258 ha commercial non-irrigated olive orchard (cv. Picual, 90 yrs old) under integrated pest management (IPM) situated in the province of Granada (37° 17′ 46.7″ N, 3° 46′ 28.7″ W), Spain. No insecticidal

treatment had been applied during the year, and natural regeneration cover was present between trees from the beginning of spring until natural drying in June. A 50 g/kaolin solution (treatment) and water (control) were applied to 16 trees (grown 11 × 11 m apart) in a square plot by means of a tractor-drawn turbo atomizer (Sistromatic AL-TAR 2000N, Máñez y Lozano S.L. Maquinaria Agrícola, Valencia, Spain) at a rate of 95 L/ha, delivering approximately 0.58 kg of kaolin per tree. Treatment and control plots were situated at a minimum distance of 80 m apart. The experiment was replicated up to four times (total 8 plots) following a RCBD with block separations of at least 150 m. Kaolin was applied twice: on 15th June and 24th Sept 2009. These dates were chosen due to the known chrysopid flying peaks in southern European olive orchards. The chrysopids were sampled seven days after the applications on two consecutive days using an insect aspirator (Modified CDC Backpack Aspirator Model 1412, John W. Hock Co., Gainsville, FL, USA) to sample all the trees in the plot. Inner and outer branches of each olive tree were suctioned up to a height of 2 m for a period of 2 min. To do this, we moved around the tree in order to cover all possible angles. The adult chrysopids collected were counted and identified at species level. Precipitation data obtained from a public agroclimatic station (IFAPA, Junta de Andalucía) revealed a single rainfall event (5.4 mm) that took place the afternoon after the kaolin treatment was carried out on 15th June and three rainfall events between 23rd September and 1st October, adding up to total rainfall of 10.2 mm.

2.9. Statistical analysis

All the statistical analysis was carried out using the SPSS Statistics 18 package for Windows (SPSS Inc., Chicago, IL, USA). To analyze larvae and egg acute mortality and larvae leaf-grasping experiments (Sections 2.2–2.4), percentages were arcsin-transformed for normalization and compared using the Student's *t*-test ($\alpha = 0.05$) for paired comparisons and analysis of variance tests (ANOVA) for multiple comparisons. ANOVA analysis was followed by Tukey tests ($\alpha = 0.05$) to identify mean differences. For larval mobility analysis (Sections 2.5 and 2.6), the parameters were $\log_{10}(x + 0.5)$ transformed for normalization and compared by means of a Student's *t*-test ($\alpha = 0.05$). In all cases, untransformed means are presented. Whenever the data distribution failed to satisfy parametric analysis assumptions, data was subjected to non-parametric Mann–Whitney *U* tests ($\alpha = 0.05$). Statistically significant differences at a confidence level of $\alpha = 0.10$ are shown. In the oviposition preference experiment (Section 2.7), as the data set violated parametric analysis assumptions, the treatments were compared using a Kruskal–Wallis test, followed by paired Mann–Whitney *U* tests for individual comparisons. The α value was adjusted by means of the Bonferroni–Holm correction ($\alpha \leqslant 0.05$) (Holm, 1979). Finally, *C. carnea* adult capture data from the field case study (Section 2.9) was analyzed using a generalized linear mixed model (GLMM) with a Poisson error distribution (R software, version 2.1.2, package glmmML). Treatment (kaolin and control) and date were used as explanatory variables and block as a random effect. No data overdispersion was detected.

3. Results

The mean particle density (±SD) deposited on the Teflon slides after drying for 4 h was 290.7 ± 99.7 μg/cm^2.

3.1. Acute mortality to larvae

No difference in mortality was observed between the treatment and control individuals (Table 1) neither at the different developmental stages (ANOVA, $F = 0.48$, df = 5, $P = 0.788$) nor in the percentage of adult emergence (*t*-test, $t = 0.42$, df = 7.97, $P = 0.685$). The clearly observable kaolin film particle covering the larvae dorsum did not interfere with *C. Carnea*'s normal development from the third instar state.

Table 1
Mean mortality (±SD) in different development stages and mean adult emergence (±SD) of third instar larvae sprayed either with water (control) or kaolin.

Treatment	% Mortality[a]			% Adult emergence[a]
	3rd Instar	Prepupa	Pupa	
Kaolin	12.0 ± 17.9	4.0 ± 8.9	10.0 ± 7.1	74.0 ± 15.2
Control	14.0 ± 11.4	8.0 ± 10.9	8.0 ± 8.4	70.0 ± 14.1

[a] Data arcsin-transformed for statistical analysis. Mean values (±SD) followed by same or no letters are not significantly different ($\alpha = 0.05$).

3.2. Acute mortality to eggs and larval survival

Egg hatching and early survival of newly emerged first instars were not affected by the kaolin treatment on eggs under the tested environmental conditions. Specifically, 80.0 ± 8.3% (mean ± SD) particle film sprayed eggs produced normally hatching individuals, while the hatching rate in water sprayed eggs was 84.3 ± 5.9%. Despite a lower hatching rate resulting from the kaolin treatment, no statistical differences were found (*t*-test, $t = 0.36$, df = 18, $P = 0.192$), which can be considered of little biological significance. No cannibalistic egg predation was observed while recording the viable and non-viable eggs, indicating that the recently hatched larvae fed on the *E. kuehniella* eggs supplied.

3.3. Effect on leaf-grasping ability

Kaolin particle film covering both sides of olive leaves clearly affected *C. carnea* larval ability to grasp the leaf surface. Their grasping capacity was altered by kaolin treatment on both the upper and lower side of the leaf (ANOVA, $F = 35.8$, df = 3, $P < 0.05$) and was especially notable in the case of the upper side, where the number of larvae that remained on the kaolin-treated surface decreased by 66%. With respect to the upper and lower parts of the leaf, no difference in grasping performance was observed when comparing water sprayed surfaces. However, the ability to grasp the treated lower surface was significantly greater than the capacity to grasp the treated upper surface (Table 2).

3.4. Effect of particle film covering C. carnea larvae on mobility and behavior

Observation of the recorded tracks did not reveal the existence of a clear behavioral change resulting from the particle film adhering to the insect's surface. Despite this, the numerical variables derived from the tracks provided by the EthoVision XT software showed small but significant differences in all of the measured parameters (Fig. 1). Kaolin-treated larvae covered a shorter

Table 2
Mean (±SD) percentage of *C. carnea* larvae able to grasp to kaolin treated and control leaf surfaces and therefore not falling within 30 s after inverting them.

Treatment	% Grasping the leaves[a]	
	Upper surface	Lower surface
Kaolin	22 ± 11 a	62 ± 16 b
Control	88 ± 11 c	92 ± 8 c

[a] Data arcsin-transformed for statistical analysis. Mean values (±SD) followed by equal letters are not significantly different (Tukey's test, $\alpha = 0.05$).

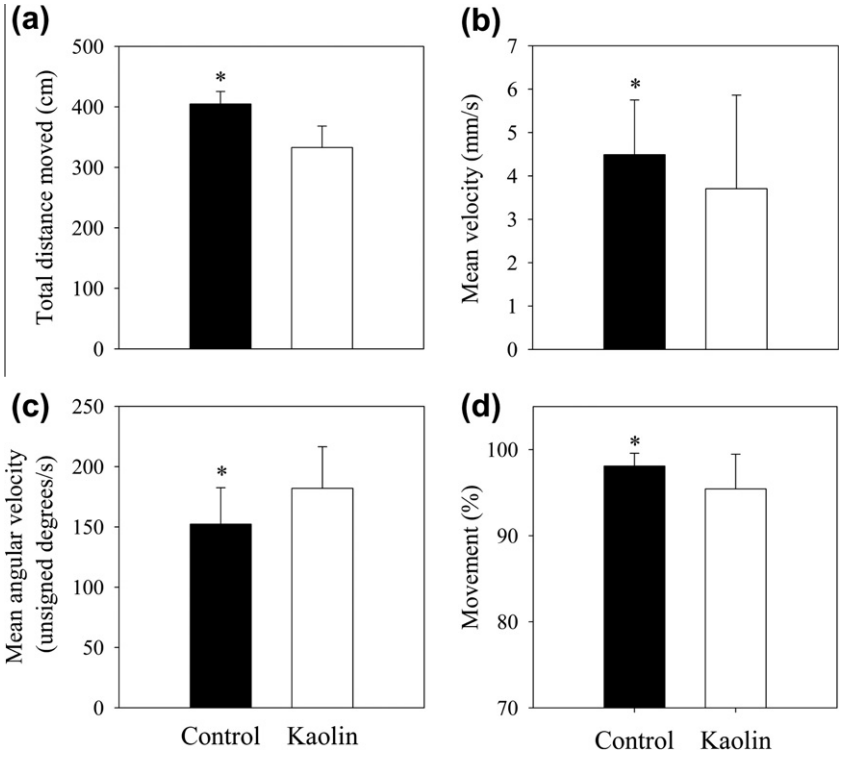

Fig. 1. Movement parameters of *C. carnea* larvae directly sprayed either with kaolin or mineral water (control). Mean ± SE (a) total distance moved (cm), (b) mean velocity (mm/s), (c) mean angular velocity (unsigned degrees/s) and (d) movement (%). Bars with different symbols indicate statistically significant differences ($P < 0.05$).

distance within the arena than water-treated individuals (*t*-test, $t = 2.60$, df = 44.23, $P = 0.012$). The reduction in the distance moved can be interpreted as both an effect produced by a decreased mean velocity of kaolin-treated individuals (*t*-test, $t = 2.56$, df = 44.59, $P = 0.014$) and an increased frequency of pausing or spending a lower fraction of the total trial time in motion (Mann–Whitney *U* test, $Z = -2.61$, $P = 0.009$). Kaolin treatment also affected the shape of the path travelled by the larvae; kaolin-covered individuals showed a significantly higher turning rate per unit of time (*t*-test, $t = 2.56$, df = 58.00, $P = 0.001$).

3.5. Effect of particle film surface on larval mobility and choice

As above, direct observation did not reveal a distinct reaction caused by the presence of a kaolin film. Unlike the results described in Section 3.3, where the larvae moved freely across both the dish and the lid, the individuals showed a recurrent trend to attempt to climb the dish edge from kaolin and control surfaces alike. Analysis of track data showed differences in movement parameters induced by the kaolin film (Fig. 2). The tested individuals covered a shorter distance (*t*-test, $t = 2.49$, df = 98.00, $P = 0.014$) at a lower velocity (Mann–Whitney *U* test, $Z = -1.66$, $P = 0.097$) on the treated surface. The larvae spent significantly less time on the kaolin film surface (*t*-test, $t = 3.78$, df = 98.00, $P = 0.007$) and showed a higher stopping frequency (*t*-test, $t = 3.01$, df = 98.00, $P = 0.007$) as compared to the control surface. No differences were detected in the path shape given that the angular velocity exhibited was similar on both surfaces (*t*-test, $t = 1.05$, df = 82.45, $P = 0.295$).

3.6. Adult oviposition preference

C. carnea female adults laid almost all the eggs upside down on the lower part of the leaves attached to the lid, while just a few were deposited on the lower side and the lateral border (Table 3). On the lower surface, where the eggs accounted for 94.9 ± 6.4% (mean ± SD) of the total, the tested individuals laid more than twice the number of eggs on the kaolin-treated semicircular zone compared with the control zone (Kruskal–Wallis, $F = 302.88$, df = 4, $P < 0.05$).

3.7. Effect on adult abundance. Field case study

From a total of 256 samples, 111 adult chrysopids belonging to four different species were captured (Table 4). *C. carnea* adults represented 91.9% of the total individuals and were the only species captured in June. Only a few *C. carnea* adults were collected on these first sampling dates (0.25 ± 1.05 captures per tree (mean ± SD) in control plots and 0.09 ± 0.42 in kaolin plots). However, the total number of *C. carnea* adults captured increased nearly ninefold in October (GLMM, $Z = 6.62$, $P < 0.01$), recording 0.86 ± 1.37 captures per tree in kaolin plots and 0.56 ± 1.10 in control plots. Overall, no significant effect of kaolin treatments on *C. carnea* adult abundance was detected (GLMM, $Z = -1.38$, $P = 0.168$).

4. Discussion

Although the kaolin-based particle film is considered to be an innocuous compound, its direct spray effect on insects varies depending on the test species. In psyllids, Hall et al. (2007) found no acute toxicity when spraying a 3% Surround WP suspension on Asian psyllid (*D. citri*) adults, nymphs and eggs. However, pear psylla nymphs and adults subjected to similar tests recorded significant mortality rates (Puterka et al., 2005). *Plutella xylostella* (L.) fourth instar mortality was just slightly increased by a particle film residual coating (Barker et al., 2006), and Larentzaki et al. (2008) reported that an interference with feeding capacity was the most probable cause of the higher mortality rates observed in kaolin-sprayed *T. tabaci*. In the case of *C. carnea*, direct kaolin

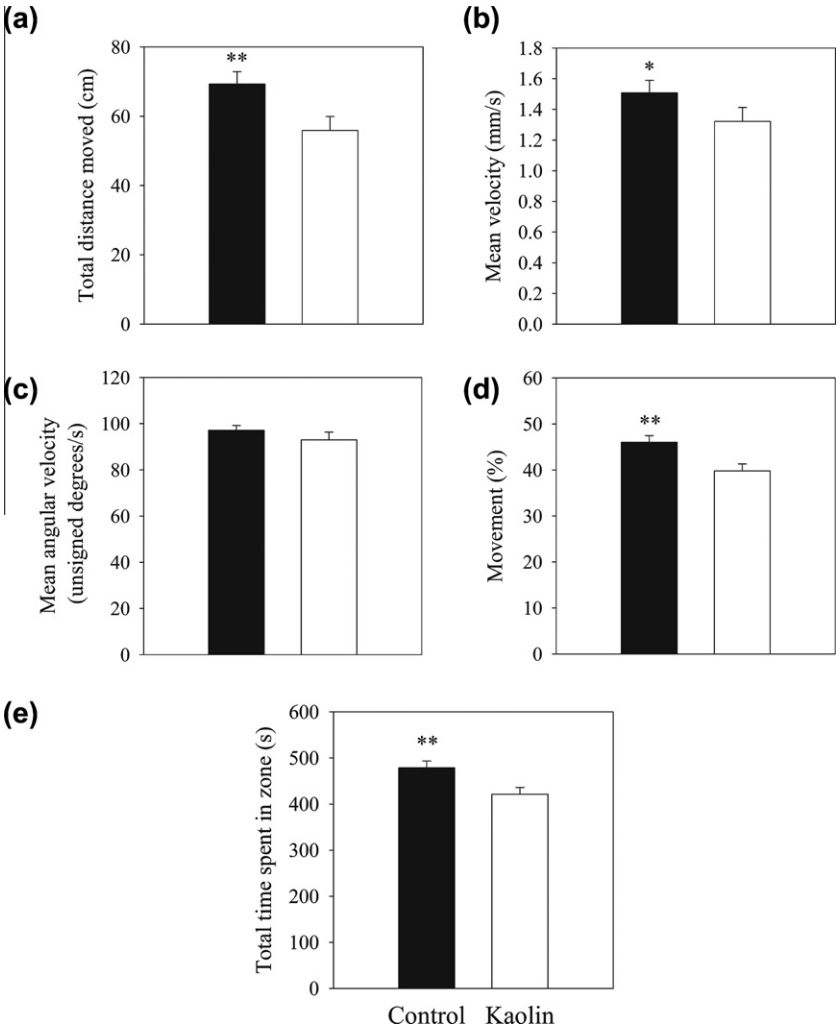

Fig. 2. *C. carnea* larvae movement parameters on a circular arena half of which is covered with a kaolin particle film and the other half kaolin free (control). Mean ± SE (a) total distance moved (cm), (b) mean velocity (cm/s), (c) mean angular velocity (unsigned degrees/s), (d) movement (%) and (e) total time spent in zone. Bars with different symbols indicate statistically significant differences $^{**}P < 0.05$; $^{*}P < 0.10$.

Table 3
Mean number (±SD) of eggs laid by *C. carnea* 7-d-old females in a 48 h time period on upper and lower olive leaves surfaces treated either with kaolin particle film or mineral water (control).

Treatment	# Eggs laid[a]		
	Upper surface	Lower surface	Lateral border
Kaolin	0.2 ± 0.7 a	17.3 ± 8.7 b	1.2 ± 1.7 d
Control	0.0 ± 0.0 a	7.3 ± 6.5 c	

[a] Mean values with different letters indicate statically significant differences (Mann–Whitney *U*-test, Bonferroni-Holm's correction $\alpha \leqslant 0.05$).

Table 4
Number of Chrysopidae adults captured by suction sampling in kaolin sprayed and water sprayed (control) olive trees in two different sampling events.

Species		June		October	
		Control	Kaolin	Control	Kaolin
Chrysoperla carnea (Stephens)	♀	5	1	19	24
	♂	3	2	17	31
Dichocrysa prasina (Burmeister)	♀	0	0	1	0
	♂	0	0	0	0
Dichocrysa flavifrons (Brauer)	♀	0	0	1	0
	♂	0	0	1	1
Chrysopa formosa Brauer	♀	0	0	0	0
	♂	0	0	2	3
Total		8	3	41	59

applications do not appear to cause any direct mortality either in terms of interfering with feeding capacity or other effects that may reduce survival. Hall et al. (2007) suggested body size as a possible explanation for the differential effect on mortality among insect species. This opens up the possibility that early *C. carnea* first instars (around 2 mm), which were not covered in this study, may be affected by residual kaolin coating. However, we have observed that ecdysis removes the kaolin coverage that remains attached to the molt. Therefore, we believe that kaolin particles have a zero, or insignificant, acute effect on *C. carnea* mortality. As for the ovicidal effect of kaolin, the results are consistent with experiments carried out on moth eggs unaffected by the kaolin cover (Barker et al., 2006; Unruh et al., 2000).

Although the kaolin residual coating did not affect mortality, the behavioral bioassay showed that kaolin-covered larvae were less mobile, slower, stopped more often, and thus travelled shorter

distances. To the best of our knowledge, no such effects of kaolin residual coating have been previously reported for other insect species. Although the reason for these differences is not known, it might be related to the accumulation of kaolin particles in the articulations hindering normal walking activity. This fact could also help to explain the increase in turning activity detected. The difficulty of maintaining a straight walking line may be caused by the kaolin residue deposited on the legs. Due to the small differences between means, these results should be interpreted with caution. We cannot be sure about the actual consequences of the observed mobility reduction at a field scale. Chrysopid movement capacity is an essential element in their foraging efficiency, since prey encounters predominantly occur at random. It would be reasonable to suggest that kaolin particles adhering to the insect's surface may negatively influence prey searching and survival and therefore the overall biological control performance of the tested species. However, at field level, kaolin particle deposits will probably never reach the density assayed in the laboratory. Highly mobile chrysopid larvae may easily find shelter, thus partially avoiding the kaolin spray. By contrast, small reductions in mobility assessed at the laboratory scale over a reduced period of time may translate into a significant effect on the *C. carnea* larva life span.

Our grasping ability results are similar to those of the pear psyllid, which fell off treated surfaces as the kaolin particles tended to break away at the insect's grasping point (Puterka et al., 2005). However, falling off did not occur in relation to psyllids on the underside of kaolin coated leaves, which is most likely due to the presence of conspicuous structures such as trichomes and stomates. Although attachment is expected to differ among insect species depending on their specific adaptations, *C. carnea* larvae seem to be affected in the same manner as psyllids. Under field conditions, grasping ability inhibition may result in an increase in the number of larvae falling from the tree canopy to the ground. *C. carnea* larvae are specifically adapted to minimizing the risk of dropping off plants. They produce an adhesive substance in their Malpighian tubules that is applied through the tip of the abdomen (Spiegler, 1962). The development of this adaptation is an indication that dislodgement from the plant represents a potential source of mortality (Rosenheim et al., 1999). The movement bioassay of larvae on a kaolin film revealed hampered locomotion on treated surfaces similar to that caused by direct spraying of larvae. These movement difficulties may induce the insect to preferably move on the control surface, thus explaining the differences in time spent in each zone. The presence of kaolin film has been reported to restrict the mobility of adult psillids (Puterka et al., 2005) and neonate codling moth larvae (Unruh et al., 2000). *C. carnea* movement limitation on kaolin-covered surfaces, unlike restrictions due to insect coating film, can be regarded as a probable scenario in orchards. Moreover, the synergistic effects of both situations, which drastically hamper the insect's ability to forage, cannot be ruled out. By observing behavioral disturbances caused by kaolin films, Unruh et al. (2000) predicted that natural enemy disruptions are most likely to affect small insects that actively forage on the surface of treated plants. Our behavioral results bear out this assertion in the specific case of the predator *C. carnea,* where computerized video-tracking technology has proved to be a valuable methodological tool.

Insect oviposition deterrence has been acknowledged as one of main effects of particle films on pest insect behavior. (Lapointe et al., 2006; Larentzaki et al., 2008). Our findings indicate, on the contrary, that gravid *C. carnea* females manifest a preference for ovipositing on kaolin surfaces. This peculiar effect has previously been noted by Medina et al. (2007), who observed increased oviposition on kaolin coated twigs as compared to the water control. Kaolin particle deposits induce a structural modification in the treated leaf surfaces that discourages certain insect ovipositions (Puterka et al., 2005). However, these modifications might elicit different behavioral responses depending on the egg species type. Consequently, *C. carnea* females may have chosen the most suitable anchoring substrate for their stalked eggs.

The literature available on the risks of kaolin treatment to beneficial arthropods is limited and inconclusive with respect to the concrete effect on chrysopid abundance. Marko et al. (2008) observed no difference between kaolin and control plots in terms of adult abundance, although the amount of lacewing larvae present was larger in kaolin-treated apple trees. Pascual et al. (2010) recorded a significantly smaller number of chrysopid captures on sprayed olive trees in one of the three years studied. However, as both larvae and adults were grouped together, the particular effects on the different life stages could not be identified. Our field experiment aimed to test the possibility that, given the results on oviposition preference, a kaolin film might exert an attractive influence on *C. carnea* adults. Even though adult chrysopids are present in olive orchards throughout the year (Campos, 2001), the small number of captured individuals on the first sampling date possibly coincided with a cyclic population decline in summer. In October, captures rose significantly, and, given that *C. carnea* males and females, contrary to previous reports, were numerically higher in kaolin plots, single kaolin treatments do not appear to affect *C. carnea* adult presence in the short term. Our results regarding adult oviposition and abundance reveal that further research is required at field level in order to clarify adult lacewing responses to this compound.

Acknowledgments

The authors would like to thank María Luisa Fernández Sierra, Damián Martínez Valdivieso and Herminia Barroso for their laboratory assistance and Dr. Víctor Monserrat for his guidance on lacewing identification. We would also like to thank Daniel Paredes, Juan Castro and the Public Company for Agricultural and Fisheries Development of Andalusia for their contributions to the field experiment carried out in the experimental orchard provided by the Junta de Andalucía. This study was grant-aided by project P07-AGR-02747 (Junta de Andalucía) and project AGL 2005-00932 (Ministry of Science and Education). Finally, we would like to thank Michael O'Shea for proof-reading the document.

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