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Functional biodiversity in different agricultural systems: methods and techniques for conservation and enhancement of ecosystem services.

Presentata da: **Serena Magagnoli**

Coordinatore Dottorato **Relatore**

Giovanni Dinelli **Giovanni Burgio**

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If all mankind were to disappear, the world would regenerate back to the rich state of equilibrium that existed ten thousand years ago. If insects were to vanish, the environment would collapse into chaos.

Edward O.Wilson

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Summary

Abstract (taken from chapter 1, paragraph 1.3 of my thesis)

The general aim of my PhD was focused on study the role of different agricultural systems in promoting functional biodiversity. In particular, the importance of habitat management techniques on natural enemy enhancement and conservation was considered at farm level with two-years samplings. Predation by polyphagous predators, which represents one of the most important ecosystem services in conservation biological control, was assessed using different approaches. In this context, molecular marker and artificial caterpillars (sentinel preys made by plasticine) were used to investigate the role of polyphagous predators in different cultivated systems.

The hypothesis of my thesis is in agreement with the general assumption that diversified agro-ecosystems are more suitable for natural enemies than simplified ones. In vegetable system, natural enemies should be also favoured by the presence of *Agro-ecological Service Crops* by means of food supply, refuges and shelters. Moreover, we hypothesized that sustainable approaches such as green manure and roller crimper reduce soil disturbance and strengthen the ecosystem services, in comparison with conventional methods.

Keywords: functional biodiversity, biological control, predation pressure, artificial caterpillars, agro-ecological service crops (ASCs), natural enemies, ASC terminations, roller crimper, green manure, Carabidae, Staphylinidae, *Bactrocera oleae*, *Aphis gossypii*.

Riassunto

Le attività di ricerca svolte durante il mio PhD sono state focalizzate allo studio dei metodi e delle tecniche per conservare e promuovere la biodiversità funzionale negli agro-ecosistemi. Campionamenti biennali, effettuati a livello di azienda, hanno permesso di valutare l'azione dei predatori polifagi nei confronti degli insetti dannosi utilizzando marker molecolari e bruchi artificiali di plastilina (dummy caterpillars).

L'ipotesi della mia tesi è in accordo con il generale presupposto secondo cui i nemici naturali sono favoriti dai sistemi agricoli diversificati. A tal proposito, un punto chiave del dottorato ha riguardato l'influenza che le "*Agro-ecological Service Crops* (ASC)" hanno nei confronti degli insetti utili e dannosi in sistemi orticoli biologici. Difatti, è ben noto come le ASC avvantaggino i nemici naturali grazie a una maggiore disponibilità di cibo e di rifugi riducendo di conseguenza le infestazioni; sebbene tale affermazione debba essere valutata caso per caso. Inoltre, abbiamo ipotizzato che tecniche agronomiche quali il roller crimper e il sovescio riducano il disturbo del suolo rafforzando i servizi ecosistemici rispetto ai metodi convenzionali.

Keywords: biodiversità funzionale, lotta biologica, pressione predatoria, bruchi artificiali agro-ecological service crops (ASCs), nemici naturali, tecniche di terminazione conservativa, roller crimper, sovescio, Carabidae, Staphylinidae, *Bactrocera oleae*, *Aphis gossypii*.

1. General introduction

1.1. The role of habitat management on functional biodiversity

The impacts of humans on Earth have increasingly escalated in the last three centuries signing the beginning of a new Era called Anthropocene. This word coined in 1873 by an Italian geologist, Antonio Stoppani, describes the influence of mankind on the environments. Human activities have increased greenhouse gases in atmosphere, contaminated ground waters and soils and caused lost of biodiversity (Crutzen, 2002).The increase of pesticides and chemical fertilizers use, observed since the advent of the green revolution, contributed to the pollution of the environments. (Tilman, 1998) affirmed: "No other activity has transformed humanity, and the Earth, as much as agriculture". However, in the recent years, even more attention focused on sustainable approaches aimed to reduce non-renewable inputs in agriculture. A real contribution in this direction comes from "Integrated Biological Control", a concept proposed by (Gurr and Wratten, 1999), which is focused on implementation of Biological Control (conservation, classical and augmentation). Integrated Biological Control is mainly focused on insect control by ecological engineering, but it follows the same principles of agroecology (Gliessman, 2014). In particular, the purpose of Conservation Biological Control (CBC) is to promote or enhance natural enemy performance by habitat or landscape management, thus reducing pest outbreaks (Landis et al., 2000; Gurr et al., 2003; Fiedler et al., 2008; Wade et al., 2008; Jonsson et al., 2012). This aim is obtained by promoting habitat manipulation and ecological infrastructure management including ecological corridors, flowering strips, beetle bank, cover crops and living mulch (Gurr et al., 2003). In many countries, these strategies have become crucial to enhance functional biodiversity for pest suppression and in Italy, during the last twenty years, we are witnessing the escalation of natural vegetation management with the agreement of local governments who funded a number of agro-

environmental schemes (Burgio et al., 2004). Crop diversification by habitat management has been considered an adaptative method to improve the resilience in agriculture, under environmental change conditions (Lin, 2010).

Recently, the new terminology of *Agro-ecological Service Crop* (ASC) was introduced to include all crops with any agro-environmental functions (Canali et al., 2015). ASCs play an important role to reduce non-renewable input in agriculture, soil erosion and nitrate leaching (Di and Cameron, 2002; Ashford and Reeves, 2003; Macdonald et al., 2005) as well as water runoff (Kaspar et al., 2001; Pimentel, 2006). ASCs may affect weed emergence, but this weed control is strictly related with the amount of ASC biomass and their rate of decomposition (Teasdale, 1996; Davis, 2010; Dorn et al., 2013; Ciaccia et al., 2015).

ASCs may protect and enhance natural enemies by providing resources (pollen and nectar), physical refugia and alternative host/prey (Landis et al., 2000; Gurr et al., 2004). This relationship between natural enemies boosting and vegetation complexity represents one of the most important keystone of agroecology (Benton et al., 2003; Tews et al., 2004).

However, many variables affect the ASC management such as the termination techniques. In this regard, in many studies ASCs were terminated by applications of herbicides (Schmidt et al., 2004; Pullaro et al., 2006; Jackson and Harrison, 2008; Gill et al., 2011; Bryant et al., 2013) and even if this termination technique is still considered the best way to control weeds in ASC systems, other more sustainable approaches are available (Creamer and Dabney, 2002; Ashford and Reeves, 2003; Dorn et al., 2013). Two efficient alternatives to herbicides are green manure and roller crimper. In the first case, the incorporation of ASCs into the soil increase the rate of nutrient release and the amount of organic matter (Cherr et al., 2006). Instead, in roller crimper approach, where cover crops are flatten with a shaped roller creating a natural layer of mulch, the reduced contact

between the dead mulch and the soil is responsible of a lower amount of nutrients (Parr et al., 2014). On the other hand, the dead mulch layer prevents the germination and the weeds emergency, improving the growth and competitiveness of the main crop (Canali et al., 2013; Ciaccia et al., 2015). Finally, weeds control could be emphasize using ASCs with allelopathic characteristics by means of secondary compounds toxic for weeds (Bhowmik, 2003; Weston and Duke, 2003; Khanh et al., 2005).

1.2. Importance of predators and methods to study prey-predator interactions

The feeding habits of predators strongly influence the natural enemy-pest interactions. Thus, while more specialised predators depend on a limited number of preys, generalist predators show a different pattern feeding on a broad range of preys. Many generalist predators assume a significant relevance in biological control of pests, as they colonize agricultural fields early in the season by feeding on alternative preys. Organic rotation can positively affect soil predator communities (Burgio et al., 2015). The better conservation of arthropod fauna in the organic system seems to be coherent also with the enhancement of organic matter; moreover spray intensity may adversely affect the carabid species richness, as proved by the lowest diversity values recorded in conventional system (Burgio et al., 2015). Therefore, it is important to consider all the component and variables linked to the communities of predators (Klemola et al., 2002; Snyder and Ives, 2003) in order to improve the knowledge on the complex scenario provided by an agroecology approach for pest control. Moreover, the variations of functional biodiversity during time (Otto et al., 2008) caused, for example by the introduction of non indigenous biological control agents make difficult the CBC evaluation, because exotic species may modify the pre-existing predator guilds (Burgio et al., 2004; Otto et al., 2008; Rondoni et al., 2014).

As already discussed before, intensive and monocultural cropping systems are in many cases more vulnerable to pests than complex agricultural habitats (Altieri and Letourneau, 1982; Altieri and Nicholls, 2004). Many studies demonstrated that natural enemies are promoted in no-till agro-ecosystems and that their density increases in presence of ASCs (Altieri, 1999; Kromp, 1999; Landis et al., 2000; Sunderland and Samu, 2000; Jackson and Harrison, 2008; Bryant et al., 2013; Depalo et al., 2016), but this tendency cannot be considered a rule. In fact, even if natural enemies are usually positively affected by habitat management, in some cases it was observed a different trend (Masiunas, 1998; Carmona and Landis, 1999; Szendrei et al., 2014). This is also confirmed by (Björkman et al., 2010) who demonstrated that in a cabbage-red clover intercropping systems, oviposition of the turnip root fly (*Delia floralis* (Fallen, 1824)) was reduced by vegetation diversity, while generalist predators decreased. In this context, a better knowledge of prey-predator interactions are crucial to interpret each result with a case by case approach.

In any agricultural systems the role of predators in the pest control is difficult to quantify or assess. Predators are active mainly during night consuming all prey quickly and without leaving any remains. Different approaches could be used in order to quantify the predation pressure and they are usually distinguished into direct and indirect approaches (Furlong, 2014; Macfadyen et al., 2015). One of the most direct approaches consists in predation assessment by exclusion cages (O'neal et al., 2005; Blaauw and Isaacs, 2012; Macfadyen et al., 2015). This method is based on the evaluation of prey abundance in presence or absence of its predators. The removal of predators from the cage experiment could occur by means of mechanical barriers, application of insecticides, hand removal and selective killing of natural enemies (Furlong, 2014).

Predation pressure can also evaluate with sentinel preys by exposition of eggs and larvae to predators (Head et al., 2005; Schneider et al., 2013). However, in this case, a massive

rearing is necessary to obtain the suitable number of eggs and/or larvae and no information about predator identity is obtained. In the recent years a new technique based on artificial caterpillars was proposed to overcome these issues (Loiselle and Farji-Brener, 2002; Gonzalez-Gomez et al., 2006; Koh and Menge, 2006; Howe et al., 2009; Low et al., 2014; Marco Ferrante et al., 2014; Lövei and Ferrante, 2016).

Artificial caterpillars are built with plasticine or modelling clay and the size, shape and colour vary in accord to the aim of the study (Lövei and Ferrante, 2016). In particular, predation pressure was evaluated by exposition of artificial caterpillars to predators in order to detect their marks on the dummy surfaces (Loiselle and Farji-Brener, 2002). Furthermore, it's necessary to highlight that this standardize method can allow to compare the rate of predation in different ecological conditions, but it lacks of the chemical cues that are normally important in prey-predator interactions (Howe et al., 2009).

Finally, an important contribution to study predation pressure comes from molecular techniques by means of qualitative and quantitative methods (Symondson, 2002). The molecular approach is based on the identification of a target prey into the gut content of predator and was used for the first time in 1946 with the aim to study the larval mosquito predation (Brooke and Proske, 1946; Furlong, 2014). Since then, significant progress has been made and even more sensitive molecular techniques are now available to study predation. Despite the evolution done in molecular sciences, these approaches remain little used in studying prey-predator interactions within agro-ecosystems (Furlong, 2014).

1.3. Aim and hypothesis

The general aim of my PhD was focused on study the role of different agricultural systems in promoting functional biodiversity. In particular, the importance of habitat management techniques on natural enemy enhancement and conservation was considered at farm level

with two-years samplings. Predation by polyphagous predators, which represents one of the most important ecosystem services in conservation biological control, was assessed using different approaches. In this context, molecular marker and artificial caterpillars (sentinel preys made by plasticine) were used to investigate the role of polyphagous predators in different cultivated systems.

The hypothesis of my thesis is in agreement with the general assumption that diversified agro-ecosystems are more suitable for natural enemies than simplified ones. In vegetable system, natural enemies should be also favoured by the presence of *Agro-ecological Service Crops* by means of food supply, refuges and shelters. Moreover, we hypothesized that sustainable approaches such as green manure and roller crimper reduce soil disturbance and strengthen the ecosystem services, in comparison with conventional methods.

In addition to this introductive part (chapter 1), my thesis was divided into five more chapters:

- **Chapter 2 (preliminary-methodological study)**

Predation pressure was assessed using a new technique based on artificial caterpillars (sentinel prey or dummy caterpillar). Although this approach was already used in other countries, this study represented the first record for dummy caterpillars in an Italian agro-ecosystem. In this regard, a number of maize fields sited in Bologna province, were investigated during two years (2014-2015) in order to understand if this technique could be considered a standardize approach for Italian crop systems.

- **Chapter 3**

On the base of the promising results obtained from dummy caterpillars in maize fields, we extended this method to analyse and quantify the predation pressure in an agroecological project (Gestione agro-ecologica per la difesa delle colture orticole in biologico

"ORTOSUP") carried-out, in an organic vegetable rotation located at C.R.E.A Horticulture Research Unit of Monsampolo del Tronto (AP, Italy). The community of soil arthropods was studied in order to assess the impact of two ASCs (barley and vetch) and two ASC termination techniques (green manure and roller crimper) on functional biodiversity. In this study, the dummy caterpillar method was extended to measure the positive impact or, conversely, the potential soil disturbance of these agroecological treatments on predation.

- **Chapter 4**

Results reported in this chapter were always obtained within ORTOSUP project, but in this case I studied the agroecological effects of two ASC termination techniques (green manure and roller crimper) on the pest (*Aphis gossypii*) and natural enemy dynamics. In particular, green manure and roller crimper techniques were compared with a synthetic biodegradable film covering (MaterBi) which represents the common method to control weeds in many organic vegetable Italian systems.

- **Chapter 5**

In this chapter, I expanded the study on the assessment of predation pressure of polyphagous predators on the "*Ocypus olens* (Coleoptera: Staphylinidae)/*Bactrocera oleae* (Diptera: Tephritidae)/olive system. Gut contents were analyzed by using molecular marker. Individuals of *O.olens* were collected in five olive groves in Tuscany region and analysed by PCRs using specific primers for *B.oleae*.

Soil conservation of olive orchard was proposed as method to increase ecological sustainability. However, despite soil predation by polyphagous predators can be also important in perennial system, the role of polyphagous predators for pest control is still poorly investigated.

All field data (Chapter 2, 3, 4 and 5) were collected with seasonal investigations occurred during two consecutive years (2014-2015) with the only exception for specimens of

O.olens sampled from October 2015 to March 2016 (Chapter 6). Molecular analysis of *O.olens* gut contents were carried out in collaboration with the Plant Phatology area of the Department of Agricultural Sciences (Alma mater studiorum - Università di Bologna) and the Institute of Life Sciences, Scuola Superiore Sant'Anna (Pisa).

Furthermore, despite most of my PhD work was performed at the Department of Agricultural Science in the Entomological areas of University of Bologna (Italy), I have also spent two months at the Department of Ecology of Debrecen University (Hungary), working on the taxonomy of staphylinids (Coleoptera: Staphylinidae) and further four months at the Department of Agroecology of Flakkebjerg Research Centre (Aarhus University, Denmark) where I improved my skills about molecular analysis of predator gut content.

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2. Assessing predation pressure by artificial caterpillars: first validation for Italian agro-ecosystems.

2.1. Abstract

In the recent years, artificial caterpillars (dummy caterpillars) are used to evaluate predation pressure in many ecosystems, but its efficacy within Italian agro-ecosystems is still unknown.

Predation pressure was evaluated in five maize fields located near Bologna (North Italy) by using artificial caterpillars built with green plasticine. Samplings occurred during two years (2014-2015) from May to August. Marks left on their surfaces were identified at higher taxonomy level (chewing insects, birds and mammals) and the frequency of attacked dummies was correlated with the activity density of carabids. The positive correlation between the frequency of attacked dummies and carabid activity density confirms this technique as a good approach for study predation pressure in Italian ecosystems. Moreover, our results are in agreement with previous studies highlighting the potentiality of this standardize technique for compare predation rates among different agro-ecosystems. Dummy caterpillars provide also information about the level of soil disturbance; an important key point with directs effects on functional biodiversity.

Keywords: maize, artificial caterpillars, predation pressure

2.2. Introduction

Domesticated about 9000 years old from *parviglumis* in Iowlands of the Central Balsas (River Valley, Mexico), maize (*Zea mays* L) has spread rapidly (Hufford et al., 2012) begun one of the most crops cultivated across the world (Leff et al., 2004; Warburton et al., 2011).

During the last century, maize yields increased markedly by means of changes in crop management and thanks to the selection of maize varieties and hybrids even more

productive (Duvick, 2005). Since the Green Revolution an heavy use of synthetic compounds such as fertilizers, pesticides and herbicides have contributed to increase yields, but with detrimental effects on environments.

In the recent years many efforts are making with the purpose to decrease the environmental impacts of agriculture, but nevertheless productivity and sustainability are still two aspects that have no easy solutions (Tilman, 1999).

Genetically modified (GM) crops could represent one of the possibilities for increase yields and simultaneously reduce pesticide applications (James, 2010). However, the environmental risk of GM crops in Europe is still controversial. For this reason, the European Food Safety Authority (EFSA) proposed a risk assessment approach based on the selection of target species in order to evaluate the impact of GM plants on environments (Arpaia et al., 2014). In this context, a EU-project AMIGA (Assessing and Monitoring the Impacts of Genetically modified plants on Agro-ecosystems) was carried out in fourteen countries (Ireland, UK, Denmark, Netherlands, Finland, Sweden, Austria, Germany, Slovakia, France, Italy, Spain, Bulgaria and Romania) chosen in relation to many factors such as the availability (commercial or for research purpose) of GM crops as well as the need to include the major variability of environmental and agroecological conditions (Arpaia et al., 2014). Potato (EH92-527-1) and maize (MON810) were selected as model crops and long-term experiments were carried out in laboratories, greenhouses and fields with the purpose to assess the potential impacts of GM crops on the environments.

One of the major objections raised against GM crops regard the impact that GM crops could have on non-target organisms. Many studies have focused on this topic with discordant results (Losey et al., 1999; Dale et al., 2002;) and for this reason a better

understanding of the GM crop effects on non-target organisms should be further deepen in future studies.

Furthermore, GM crops could affect natural enemies by changing the quality and the supply of preys and hosts (Schuler et al., 1999). Beyond this aspect, it's well recognized that intensified agro-ecosystems that are maintained in a disturbed status are more susceptible to pests than diversified systems (Tilman, 1999). In this scenario, also natural enemies could be negatively affected by simplified systems due to the lack of alternative prey/host, shelters and resources as a pollen or nectar for adults. Predation plays an important ecological function to control pest outbreaks, but a complete evaluation remains still difficult to perform. Many methods, including qualitative and quantitative, were proposed to evaluate biological control by predators and parasitoids (Macfadyen et al., 2015). A new approach to assess predation rate and based on artificial caterpillars (or dummy caterpillar method), built with green plasticine was suggested (Marco Ferrante et al., 2014; Howe et al., 2015). Dummies are exposed to the predators for 24 h and then collected to detect the bites left by predators on their surface. The purpose is to obtain a standardize method to compare the predation pressure in different habitats (Lövei and Ferrante, 2016). Moreover, the rate of predation is usually considered underestimated due to the lack of chemical cues that are normally important in prey-predator interactions. However, when low densities of preys occurred, generalist predators could be responsible for the increase of predated dummies. Taking into account these considerations data collected with dummy caterpillar method should be evaluate with a case-by-case approach.

Within the partner involved in EU-project AMIGA, four countries (Denmark, Spain, Italy and Romania) have used dummy caterpillars to assess predation pressure in GM fields, including commercial non-GM systems. In the present paper the dummy caterpillars method was utilized in different commercial maize fields with the intent to understand if

this approach could be represent a standardized method to assess predation pressure in Italian scenario.

The method, validated in Italian maize fields in the perspective of an environmental risk assessment of Bt-crops, could be a suitable method to detect predation pressure also in other fields of investigation, like habitat or landscape management (see chapter 3), selectivity of control methods against pests and in general to quantify conservation biological control by predators.

2.3. Materials and methods

2.3.1.1. Site, experiment and treatments

The study was carried out in five fields, covering two consecutive seasons; information about fields were reported in table below (table 1). All maize fields were managed with IPM method.

Tab. 1 Information about maize fields sampled during both years (2014-2015)

Field	Year	Site	Coordinates	Size field (ha)	N° of pitfall stations per field	Total N° of dummy caterpillars
AGR1	2014	Cadriano	44°33′18.44" N 11°24′51.00" E	0.50	10	720
AGR2	2014	Cadriano	44°33′01.01" N 11°24′38.20" E	0.60	12	864
CAS3	2014	S. Pietro in Casale	44°40′54.02" N 11°25′34.78" E	0.50	10	640
CAD1	2015	Cadriano	44°32′54.01" N 11°24′54.54" E	0.60	12	480
CAD2	2015	Cadriano	44°32′59.10" N 11°24′38.74" E	0.51	10	576

Maize fields in Cadriano

Cadriano experimental farm belongs to University of Bologna. In this farm 2 fields were selected in 2014 and other two fields in 2015. Maize (*Zea mays* L cv AGN 583) was fertilized with 500 Kg/ha of urea added to the soil in two applications. Primagram® Gold was used as pesticides, while Ercole® was applied to control weeds.

Maize field in San Pietro in Casale

Maize (*Zea mays* L cv DKC7677) was fertilized with 450 Kg/ha of urea added to the soil in two applications. Force® was used as pesticide and a mix of terbuthylazine with sulcotrione and metolachlor was applied to control weeds.

2.3.1.2. Soil arthropods sampling

Soil arthropods were monitored from May to August with pitfall stations consisting in two plastic pots (volume 600 ml with 10 cm in circumference) connected with a plastic barrier and filled with 200 ml di glycol propylene (40%). Glasses were covered with lids in order to preserve the fallen arthropods. The stations were left in field for all the sampling period but they were activated only one week per month. Traps content were removed, moved to the laboratory and kept in refrigerator until the next sorting operation. Arthropods were divided into different groups and preserved in 70% ethyl alcohol. In particular, from the different range of collected arthropods we focused only on carabids for their important role as bioindicator (Rainio and Niemelä, 2003).

2.3.1.3. *Dummy caterpillars method*

Dummy caterpillars are artificial caterpillars made by green plasticine and in the recent years they are used as a standardize method to compare the predation rate in different habitats. Each dummy (2 x 0.3 cm) was obtained by a modified garlic press (Smeedi plus, V. nr. 776609, Denmark) and exposed to the predators for 24 hours. In total 3280 dummies were put in field, half of these were glued on a stick of bamboo and placed on the ground

near to the stock, while the other half was directly glued on maize leaves at one meter height from the ground (fig.1). Disposal scheme of dummy caterpillars is reported in fig 2. After that time dummies were collected, brought to the laboratory and then checked under binocular microscope to find any marks left by predators on their smooth surfaces. Despite predator identity to species level is not easy to detect, some features are peculiar of each predator group. For example, mammals leave on the smoothly surface of dummies the sign of their teeth, whereas birds are responsible of pecks, v or u-shaped, occurred on both side of dummy. More complex is the detection of etches, scratches, holes and stabs left by chewing insects on dummy surface. In this regard, (Low et al., 2014) have collected many marks from different predators with the purpose to create a useful guide for the identification of marks. In fig.3 is possible to see the most common marks found during my PhD. Each mark was assigned to the right predator group (chewing insects, mammals, birds) on the base of its size and shape. Moreover, in case of repeated attacks from the same predators we considered that as a single event of predation.

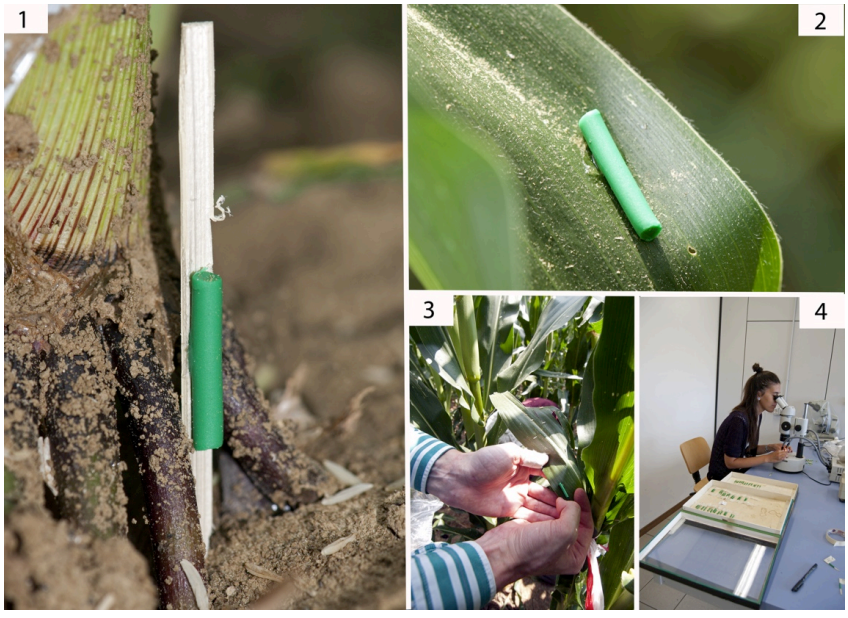

Figure 1 Dummy caterpillars were placed on the ground (1) and on maize leaves (2-3). After 24 hours dummies were brought to laboratory and checked under binocular microscope (4).

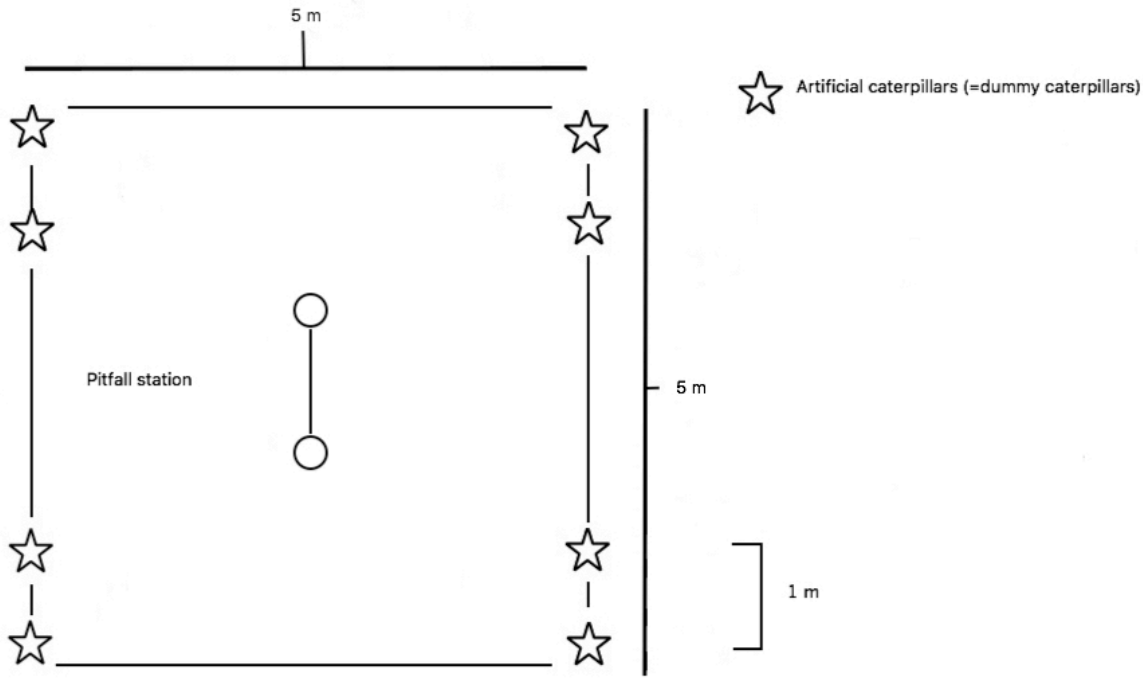

Figure 2 Disposal scheme of dummy caterpillars on the ground in each pitfall station (n=8). The same disposition was used also for dummies on leaves (n=8), but they were placed at 1 meter height.

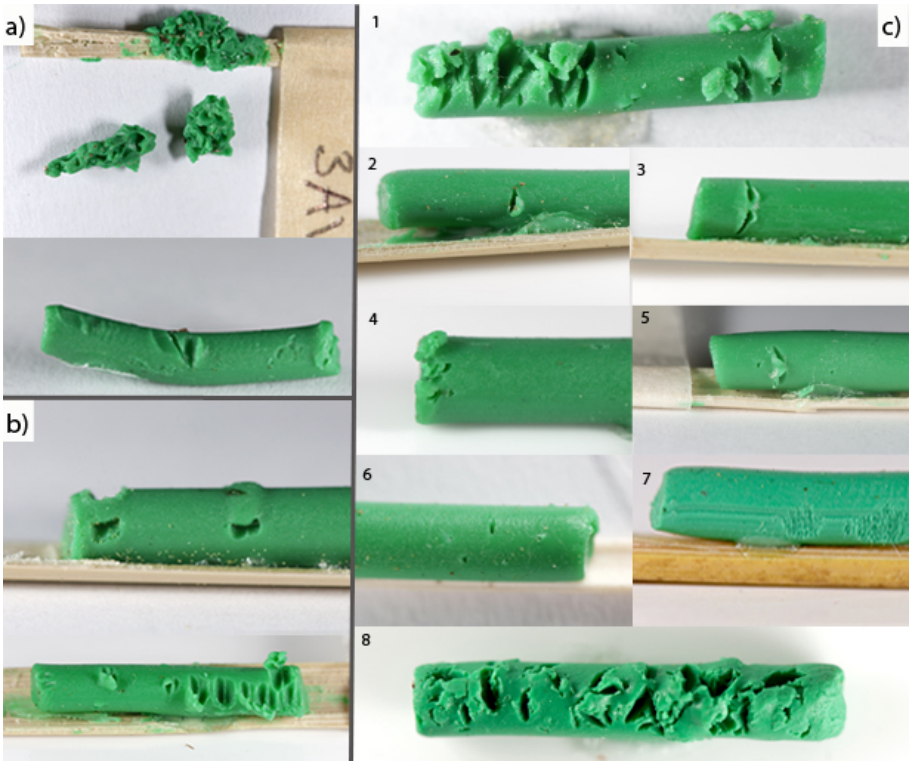

Figure 3 Different marks left on dummies: a) birds, b) mammals and c) arthropods (1-6 chewing insects, 6 spiders, 7 ants and 8 wasps).

2.3.1.4. Data analysis

Activity density was calculated with the formula reported below:

$$\frac{Number\ of\ individuals}{Number\ of\ pitfall\ traps} \text{ X} \frac{7\ days\ ^{*}}{Days\ of\ pitfall\ traps\ activation}$$

*Data were reported to a standard period of 7 days.

Spearman's rank correlation was used to assess the associations between predation rate of dummies and the activity density of medium-large (>15 mm in length) carabids.

Log linear analysis (Haberman et al., 1976) was used to test the interaction between design variables (treatments-sampling dates-years) and the response variables (dummy caterpillars predation), by a multi-way contingency table. The final matrix was based on the frequency of events of predation.

2.4. Results and discussion

Overall, 3280 dummy caterpillars were exposed to predators, half on the ground and half on the leaves. Throughout all the sampling period only 16 dummies were lost.

The rate of predation pooling dummies on the ground and on leaves (2014-2015) was 8.15% (n=266) of the total. In detail, predation of dummies on the ground was 13.47%, while predation of dummies on leaves was 2.20% (fig.4). On the 266 predated dummies the 87.22% were attacked on the ground, while only the 12.78% on leaves. The frequency distribution of attacked dummies on the ground and leaves, pooling 2014 and 2015 seasons, was reported in fig.5. In particular, chewing insects were responsible of most of the marks (74.81%), followed by birds (14.66%) and mammals (8.27%). Only in 2.66% of

cases it was not possible identify the marks left by predator, and they were classified as "unknown".

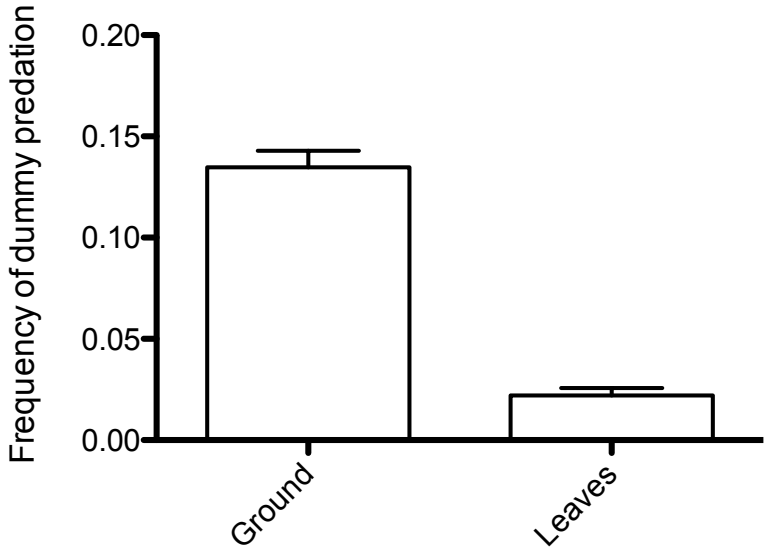

Figure 4 Frequencies of predation of dummy caterpillars on ground and leaves by pooling fields (n=5), sampling dates (n=8) and years (n=2). Bars represent SE of binomial distribution.

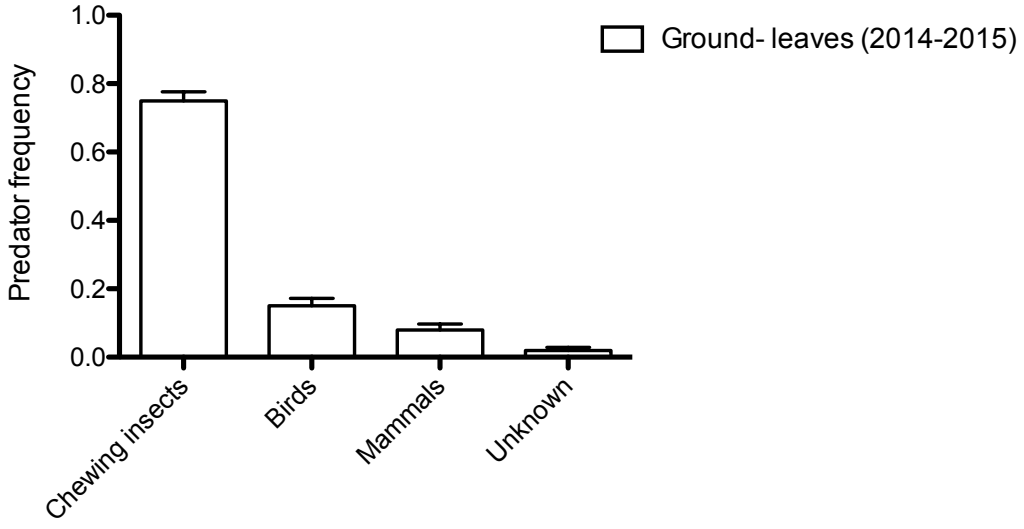

Figure 5 Distribution of the predation among the groups responsible for the marks left on dummies in both years (2014-2015). Bars represent SE of binomial distribution.

Activity density of carabids (>15 mm in length) was positively correlated with mark frequencies of chewing insects (fig.6) pooling all the sampling dates and years (fig.7 and

8). Log linear analysis demonstrated significant interaction of predation (response variable) with fields (design variable) in each season (year 2014: chi-square=12.215, d.f.=2, P<0.05; year 2015: chi-square=13.774, d.f.=2, P<0.005).

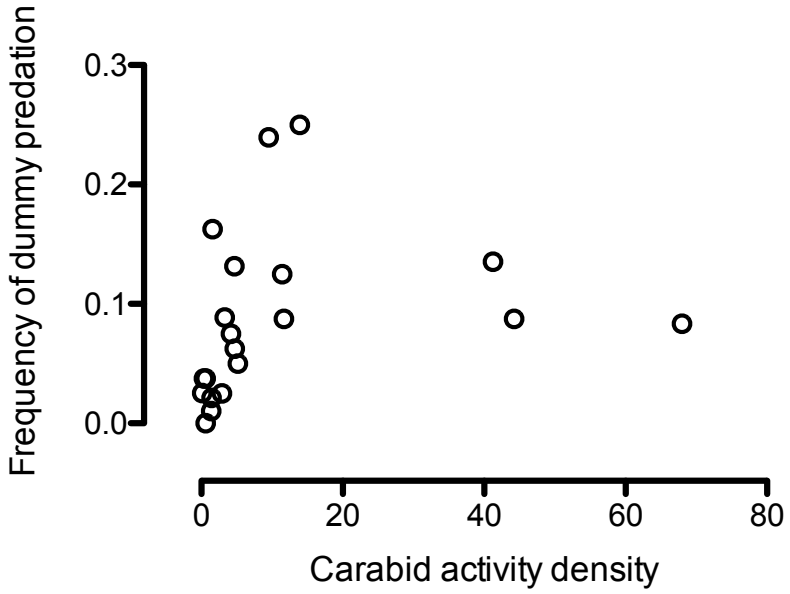

Figure 6 Correlation between carabid activity density with body size >15 mm and frequency of dummy predation by chewing insects (Spearman's rank correlation; R=0.6569, P<0.005).

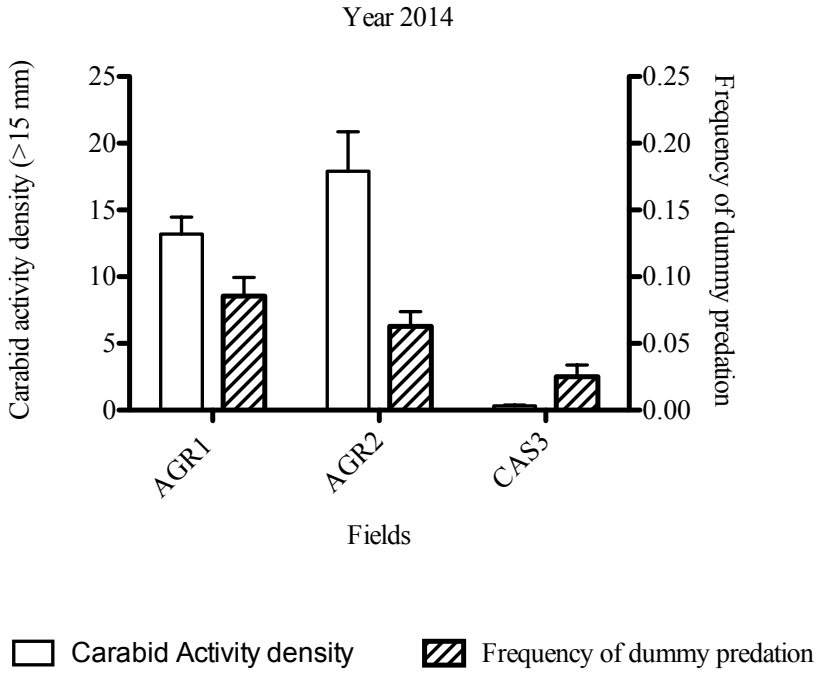

Figure 7 Carabid activity density (bars represents SE of mean) and frequency of dummy predation (bars represent SE of binomial distribution) by chewing insects during 2014.

Fields were different between the two years investigated due to crop rotation.

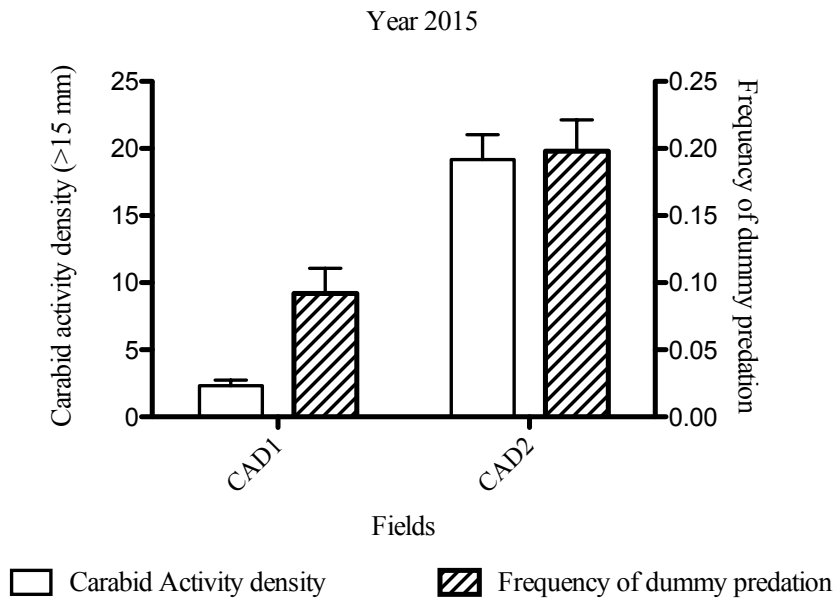

Figure 8 Carabid activity density (bars represents SE of mean) and frequency of dummy predation (bars represent SE of binomial distribution) by chewing insects. Fields were different between the two years investigated due to crop rotation.

The variability of predation among fields could be caused by many factors, including: i. landscape composition, ii. climate conditions, iii. species reproductive period of carabid species and also iv. field management. The influence of the landscape complexity on carabid abundance was pointed out by many studies (Bommarco, 1998; Tscharntke et al., 2007; Gardiner et al., 2010). However, despite these considerations, the purpose of this chapter was not focus on understanding which factors have influenced the carabid activity density among fields, but figure out if dummy caterpillar method was a sensitive technique to assess predation rate.

Given that, this is the first time that artificial caterpillars were used in Italy, we decided to validate this approach before use it in other Italian agro-ecosystems (see chapter 3). The suitability to acquire information about both predation rate and predator identity makes artificial caterpillars a potential approach to compare predation pressure in different agro-

ecosystems. Furthermore, dummy caterpillars don't require particular equipment for their realization and the material is inexpensive and easy to find and manage (Howe et al., 2009). In conclusion, on the base of previous results, we decided to utilize the same approach (see chapter 3) also within a funded project (ORTOSUP-Gestione agro-ecologica per la difesa delle colture orticole in biologico) in order to assess by dummy caterpillars the predation rate and the soil disturbance in an organic vegetable system in Monsampolo del Tronto (Central Italy) characterised by two cover crops and different termination techniques.

2.5. Acknowledgement

This study was carried out within the AMIGA project (Assessing and Monitoring the Impacts of Genetically modified plants on Agro-ecosystems) funded by European Commission. I would like to thank Dott. Gabor Lövei and Dott. Marco Ferrante for their help with dummy caterpillars method.

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3. Predation pressure on artificial caterpillars under different soil management techniques

3.1. Abstract

We studied the impact that two *Agro-ecological Service Crops* (ASC), vetch and barley, and two different ASC termination techniques (using a roller crimper and green manure) had on predation pressure in an organic vegetable system. The two ASC termination techniques were compared, during two consecutive growing seasons (2014-2015), with a biodegradable plastic mulched control that is the method commonly used to control weeds in vegetable organic systems.

Predation pressure was evaluated by using artificial caterpillars (dummy caterpillars) made of green plasticine. Marks left on their surface were assigned to higher taxonomy ranks distinguishing among chewing insects, birds and mammals. The frequencies of attacked dummies were significantly correlated with the activity density of carabids.

Predation rate was significantly higher in the field with vetch flattened by roller crimper, while no significant differences were found when barley was used as an ASC.

The rate of ASC decomposition was higher in vetch than in barley and this could be the reason for the higher activity density of springtails. Furthermore, carabid activity density was positively correlated with that of springtails.

Likely, the rate of vetch decomposition influenced positively springtails and their presence had an important role in supporting generalist carabid predators. In conclusion, our field experiments showed that dummy caterpillars could represent a good standardized method to assess predation pressure in different habitats. Moreover, ASCs and ASC terminations had an important role to reduce soil disturbance and improve soil quality by ASC decomposition.

Keywords: cover crops, roller crimper, artificial caterpillars, predation pressure.

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3.2. Introduction

In conservation biological control, natural enemies can be protected and enhanced by habitat manipulation (Gurr et al., 2017). The importance of polyphagous predators in pest control is widely accepted, though their activity is difficult to detect and measure. Most of predators forage during night, consuming directly a number of preys without leaving any remain, thus hindering direct observations of the outcome of predation (Lövei and Ferrante, 2016). Agricultural practices can influence the abundance and the activity density of soil arthropods in many cropping systems (Altieri, 1999; Miñarro and Dapena, 2003; Burgio et al., 2014) and in this context carabids are one of the most frequently studied group (Rainio and Niemelä, 2003). Evaluation of the ecological impact of habitat and landscape management on soil-living predators can made by means of faunistic analysis and the use of bioindicators. This widely used method is an important step to assess sustainability of practices, but it does not allow the quantification of the intensity of ecosystem services like predation. For this reason, practical methods to evaluate the sustainability of soil practices require a direct estimation of ecosystem services, including biological control.

Several methods are available to evaluate the impact of beneficial arthropods on pests, including predators (Macfadyen et al., 2015). Exclusion cages can be used in order to estimate the impact of a predator on pests and to study the prey-predators interaction in the trophic webs. Other methods of predation detection involve gut dissection, use of serological methods like ELISA analysis and the use of molecular markers (Symondson,

2002). Another technique is to use group of sentinel preys (eggs and larvae) exposed to predators. The rate of disappearance of such prey indicates predation intensity (Lövei and Ferrante, 2016). Such prey does not have to be real; artificial plasticine caterpillars (dummy caterpillars) has been proposed as standard and practical methods to measure the intensity of predation; also, the analysis of the marks left by predators on dummies can provide information about the identity of predators (Howe et al., 2009; Low et al., 2014).

Recently, dummies have been utilised as standardize method to compare the rate of predation in different habitats. (Loiselle and Farji-Brener, 2002; Gonzalez-Gomez et al., 2006; Koh and Menge, 2006; Posa et al., 2007; Howe et al., 2009; Ruiz-Guerra et al., 2012; Tvardikova and Novotny, 2012; Low et al., 2014; Marco Ferrante et al., 2014). However some authors (Koh and Menge, 2006; Tvardikova and Novotny, 2012) pointed out that by this method predation can be underestimated due to the lack of the chemical cues involved in the attraction of natural enemies

In this study, we investigated the impact on polyphagous predators of two *Agro-ecological service crops* (ASC), vetch (*Vicia villosa* Roth) and barley (*Hordeum vulgare* L.) and two ASC termination techniques (roller crimper and green manure). The new terminology of Agro-ecological Service Crops (ASC) has been introduced with the purpose to include all the crops (i.e. cover crops, living mulches and catch crops) with any environmental function (Canali et al., 2015). In this context, ASCs represent an important ecological approach that can be used both to enhance natural enemies and to reduce non-renewable input in agriculture. ASCs improve the vegetation complexity within agro-ecosystems leading to a positive impact on natural enemies abundance by providing resources such as nectar and pollen, physical refugee and alternative host/prey to beneficial insects (Landis et al., 2000; Gurr et al., 2004). Moreover, ASCs play an important role in weed control as well as in reduce soil erosion and leaching losses of nitrogen (Ciaccia et al., 2015).

However, all those benefits are strictly related with the choice of ASC and its termination technique. In this regard, it's well known that nitrogen is one of the major limiting nutrients for plant growth and its availability is related, in addition to nitrogen fixation, with the C/N ratio of plant (Franche et al., 2009). Leguminous ASC, such as vetch, are able to increase the nitrogen availability in soil, both for their relationship with symbiotic bacteria and also for the low C/N ratio (Eiland et al., 2001). In fact, in presence of low C/N ratio microorganisms can decompose ASC more efficaciously than plant with high C/N ratio improved the nitrogen mineralization.

In this study, dummy caterpillars method was used in order to assess both predation rate and the ecological sustainability of different soil management techniques. Overall, this quantification of predation by dummy caterpillar was employed for the first time in Italy (Lövei and Ferrante, 2016).

In order to evaluate if dummy caterpillars method represents an efficient approach to assess predation in an organic vegetable system, we correlated frequencies of predated dummies with the activity density of carabids in each experimental system. Our hypothesis is that the frequency of predation of the artificial caterpillars by a predators (i.e. carabids), if correlated to their activity density, could be used as standard method to compare the sustainability or, conversely, the disturbance of agroecological techniques.

3.3. Materials and methods

3.3.1.1. *Site, experiment and treatments*

Field experiments were conducted at C.R.E.A Horticulture Research Unit of Monsampolo del Tronto in Central Italy (latitude 42° 53′ N, longitude 13° 48′ E). Two fields (21x24 m) with different cash crop (tomato and zucchini, respectively) and ASCs (vetch and barley, respectively) were established during two growing seasons (2014 and 2015).

Each field was split into three treatments of equal size (8x21 m) on the base of the different techniques used to control weeds during our study (fig.1). In particular, two ASC termination techniques, roller crimper (RC) and green manure (GM) were compared with a control characterised by a synthetic biodegradable film cover (MaterBi, MB) which is the usual techniques used to control weeds in vegetable crops in Central and Northern Italy. The mulch layer was obtained by roller machinery provided with a shaped roller; a sharp vertical disk and a coulter were installed at front and rear of the roller respectively (fig.2).

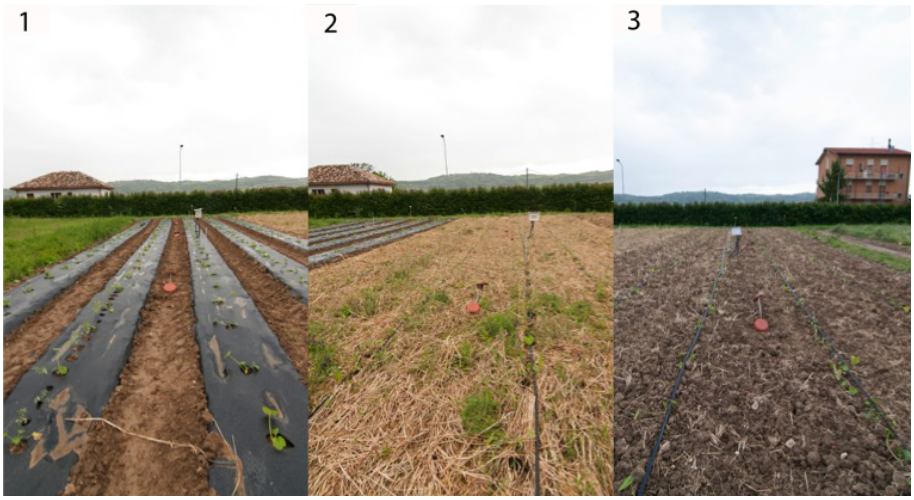

Figure 1 Each field (vetch-tomato and barley-zucchini) was split up into three treatments of equal size on the base of the different techniques used to control weeds during our study (1= synthetic biodegradable film covering (MB), 2= roller crimper (RC), 3= green manure (GM).

Figure 2 Roller crimper machynery provided with a shaped roller to flatten ASC.

In each experiment, the choice of ASC was decided taking into account the cash crop in the rotation; for this reason, tomato and zucchini were selected after vetch and barley respectively, on the basis of previous results obtained in the same cultivation area (Teasdale and Abdul-Baki, 1995; Campanelli and Canali, 2012). In particular, vetch was selected as cover crop for tomato due to its elevated content of nitrogen. This aspect is in line with the higher nutrient requirements of this crop compared to zucchini (Hartwig and Ammon, 2002).

Experiment 1 (vetch and tomato)

In the first experimental area, vetch (*Vicia villosa* Roth. - 80 kg/ha) was used as ASC. It was sown on 27 September 2013 and 24 September 2014. Cover crop termination occurred on 30 April in both years. The cash crop tomato was transplanted (2.2 plants/m^2, cultivar SAAB_CREA) on 6 May in 2014 and 8 May 2015. The fungicide Cuproxat SDI (active ingredient: 15.2 % of tribasic copper sulphate; dose: 400 ml/hl) was applied on cash crop twice in 2013 and once 2014. The irrigation rate was 4 hl/ha with plants at the beginning of their vegetative growth and 6 hl/ha in the last period of the growing season.

Experiment 2 (barley-zucchini)

Barley (*Hordeum vulgare* L. - 200 kg/ha) was sown on 31 October 2013 and 21 October 2014. Cover crop termination occurred on 30th April in both years. The cash crop, zucchini was transplanted (0.83 plants/m^2, cv. Zuboda) on 7 May in 2014 and 6 May 2015.

No pesticides, insecticides nor herbicides were applied during both growing seasons in either experiment. The fertilization rate was the same in both fields and years: N (50 kg/ha), P_2O_5 (13 kg/ha) and K_2O (21 kg/ha).

3.3.1.2. *Soil arthropod sampling*

Arthropods were monitored fortnightly by pitfall stations from early June to the beginning of August. Each station consisted of two plastic glasses (volume of 600 ml with 10 cm in diameter) filled with 200 ml of 40% propylene glycol and connected with a 0.2 x 1 m plastic barrier. Pots were buried at ground level and covered with plastic lids in order to hamper to scavenger vertebrates to falling in the traps and to prevent flooding by rainfalls. In total three pitfall stations, 8 meters apart from each other, were established per treatment. Traps were continuously activated during the two monitoring seasons, but were served fortnightly. Traps contents were removed to laboratory and kept at 4°C until sorting.

Arthropods were divided into different groups (carabids, spiders, rove beetles and springtails) and preserved in 70% ethyl alcohol. From the catch, we selected only carabids (Carabidae) and springtails (Collembola). Carabids, which represented one of the most abundant taxon in our experiment, were selected for their sensitivity to anthropogenic changes in habitat quality (Kromp, 1999). In this regard, during our study, their responses allowed to compare the sustainability or, conversely, the disturbance of the agroecological techniques used to control weeds. Moreover, carabids were identified to species level, using dichotomous keys (Pesarini and Monzini, 2010; Pesarini and Monzini, 2011) and the activity density of carabids with body length major than 15 mm were correlated with the frequency of dummy attacked by chewing insects. Likely, dummy caterpillars are more suitable to predation by carabids > 15 mm than smaller one.

Springtails were also monitored and considered in data analysis for their important role in terrestrial food webs being one of the most important prey groups for many generalist predators. Pitfall trap is not considered the most suitable technique to sample this group because their abundance could be underestimated in comparison other techniques, such as

litter bags (Prasifka et al., 2007); moreover also different species occurrs in relation to the sampling method (Querner and Bruckner, 2010). Notwithstanding, springtails were collected in high number during the samplings and they were considered in data analysis.

3.3.1.3. *Dummy caterpillars method*

Dummy caterpillars are artificial caterpillars made of green plasticine (Smeedi plus, V. nr. 776609, Denmark). Caterpillars (2x0.3 cm) were obtained by a modified garlic press. For each pitfall station a total of a 16 dummies on ground was set, for a total of 144 dummies per experiment. Eight of the dummies were glued (Super Attak Power Flex, Loctite) on a small piece of bamboo and placed on the ground, next to the plant stem; the other eight dummies were directly glued on leaves. Dummies were exposed to predators every fifteen days for a period of 24 h, collected and examined under binocular microscope, to detect any predator marks. The identification of marks was done at coarse taxonomic level, given that the identification at species level could be erroneous (Low et al., 2014) For this reason, shape and size of each mark were analysed and assigned to the following predator groups: chewing insects, mammals and birds (see chapter 2 for more details about marks identification). Repeated predator marks by the same predator, were considered as a single attack, while marks by different predators on the same dummy caterpillar were accounted as independent events of predation.

3.3.1.4. Data analysis

Activity density of carabids was calculated with the formula reported below:

$$\frac{Number\ of\ individuals}{Number\ of\ pitfall\ traps} \mathrm{X} \frac{7\ days\ ^{*}}{Days\ of\ pitfall\ traps\ activation}$$

*Data were reported to a standard period of 7 days.

Spearman's rank correlation was used to test the associations between activity density of large (>15 mm body length) carabids and frequency of dummy attack by chewing insects.

Log linear analysis (Haberman et al., 1976) was used to test the interaction between design variables (treatments, sampling dates and years) and the response variables (frequency of dummy caterpillars predation), by a multi-way contingency table. The final matrix was based on the frequency of events of predation.

The significant interactions between predation (%) and the design variables with more than 2 levels were tested by Chi square test followed by a z-test to separate column proportions for each row of the contingency table. P-values were adjusted by the Bonferroni method (Sharpe D, 2015). Software packages SPSS 20 (IBM) and Statistica (Statsoft Italy) were employed to run the statistical analyses.

3.4. Results

In total, 2592 dummies were placed in the field experiment. Predation marks were detected only on artificial caterpillar located on ground, whereas no predation occurred on dummies on leaves. For this reasons, they were excluded in the further analysis. Overall, only four dummies put on the ground were lost during our study.

Experiment 1 (vetch-tomato)

The results of predation rate in each conservation technique are reported in fig. 3, while in fig.4 the variation of the predation rate in each season is shown. Arthropods were responsible for most of the marks left on dummies (fig.5).

Log linear analysis (tab.1) showed significant interactions of predation (response variable) with: i) treatment ($P<0.001$), ii) year ($P<0.001$) and iii) sampling dates ($P<0.001$). In RC treatment, predation was significantly higher than in GM and MB, as confirmed by Chi-square test followed by a z-test (chi-square=21,152; d.f=2; $P<0.001$).

Predation rates was higher in 2015 than in 2014 (significant interactions "year*predation", chi-square=17.88; d.f.=1 P<0.001); a significant interaction was observed among "year*sampling dates*predation" (chi-square=22.03; d.f.=4; P<0.001).

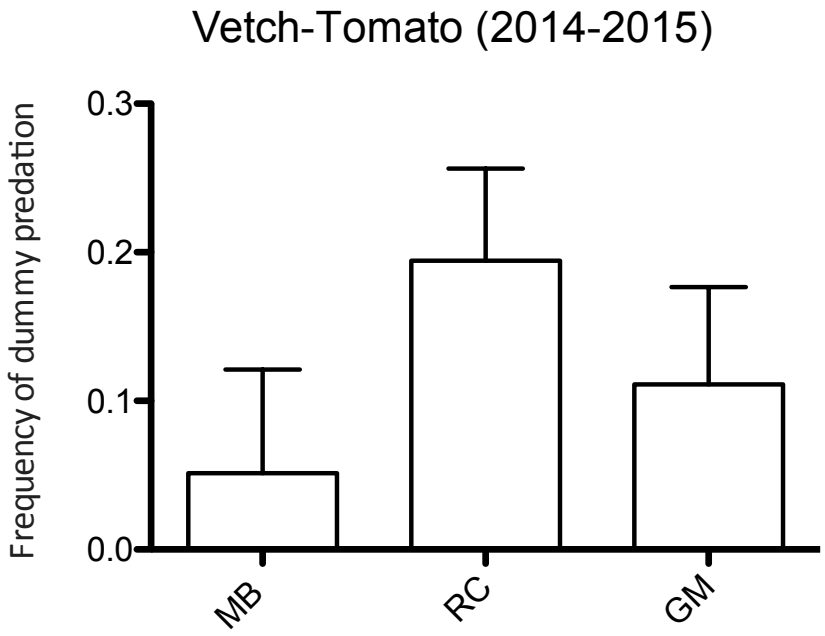

Figure 3 Frequency of dummy predation in the experiment 1 (vetch-tomato) per each treatment (MB=synthetic biodegradable film covering, RC=roller crimper, GM=green manure), pooling the seasons (2014-2015). Bars represent SE of binomial distribution.

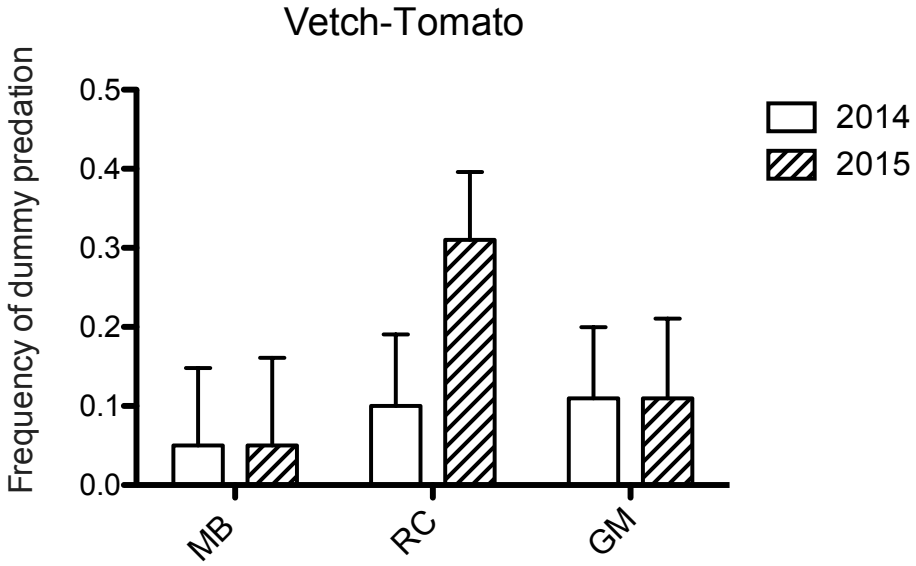

Figure 4 Frequency of dummy predation in experiment 1 (vetch-tomato) for each year

(MB=synthetic biodegradable film covering, RC=roller crimper, GM=green manure). Bars represent SE of binomial distribution.

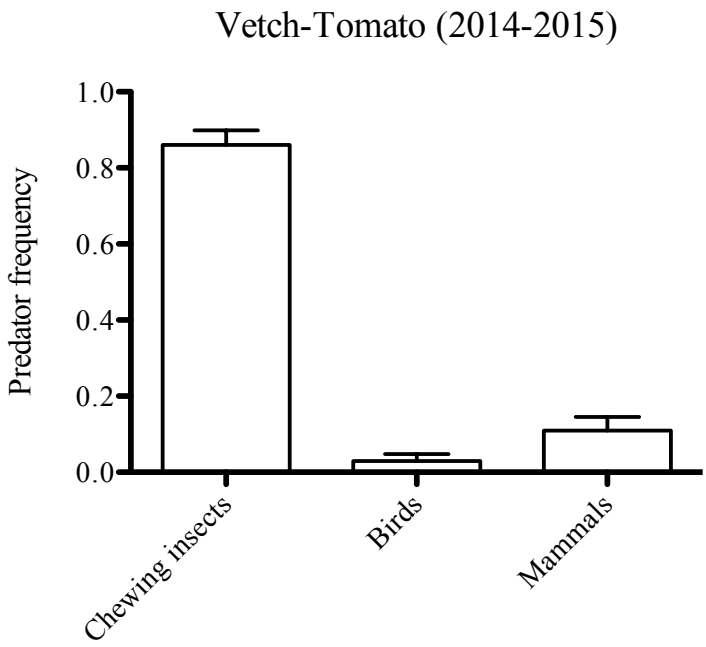

Figure 5 Distribution of the predation among the groups responsible for the marks left on dummies in experiment 1 (vetch-tomato) during both years (2014-2015). Bars represent SE of binomial distribution.

Table 1 Results of the Log linear analysis, showing the interactions between the design variables (treatment-sampling date-year) and the response variables (predation) in experiment 1 (vetch-tomato).

Effect	**df**	**$\chi 2$**	**P**
Treatment*Predation	2	20.85	<0.001
Year*Predation	1	17.88	<0.001
Sampling date*Predation	4	28.29	<0.001
Treatment*Sampling Date*Predation	8	11.11	>0.05
Year*Treatment*Predation	2	4.88	>0.05
Year*SamplingDate*Predation	4	22.03	<0.001

In both years, activity density of carabids was higher in ASC plots than in MB in both years (tab.2 and fig. 7 and 8); in this experiment, carabids were significantly correlated

with the activity density of springtails, pooling the two ASC termination techniques, (Spearman's rank correlation; R=0.5029, P< 0.0001 (fig.6)). In table 2 is also reported the activity density of all arthropod groups sampled during both years.

Table 2 Mean arthropod activity density (SE) in experiment 1 (vetch-tomato) during 2014 and 2015; MB=synthetic biodegradable film covering, RC=roller crimper, GM=green manure.

Year	Treatments	Carabids	Springtails	Spiders	Rove beetles
2014	MB	1.21 (0.78)	1.34 (0.62)	2.57 (1.21)	0.40 (0.39)
	RC	5.82 (3.29)	10.25 (4.13)	10.51 (7.12)	1.50 (0.70)
	GM	3.49 (0.93)	7.46 (3.13)	4.37 (1.48)	1.53 (0.63)
2015	MB	3.37 (1.43)	21.06 (7.96)	5.93 (4.05)	1.07 (0.84)
	RC	18.38 (5.84)	51.11 (40.73)	15.29 (4.31)	4.60 (1.70)
	GM	16.36 (3.39)	46.51 (33.02)	13.40 (3.86)	6.02 (2.96)

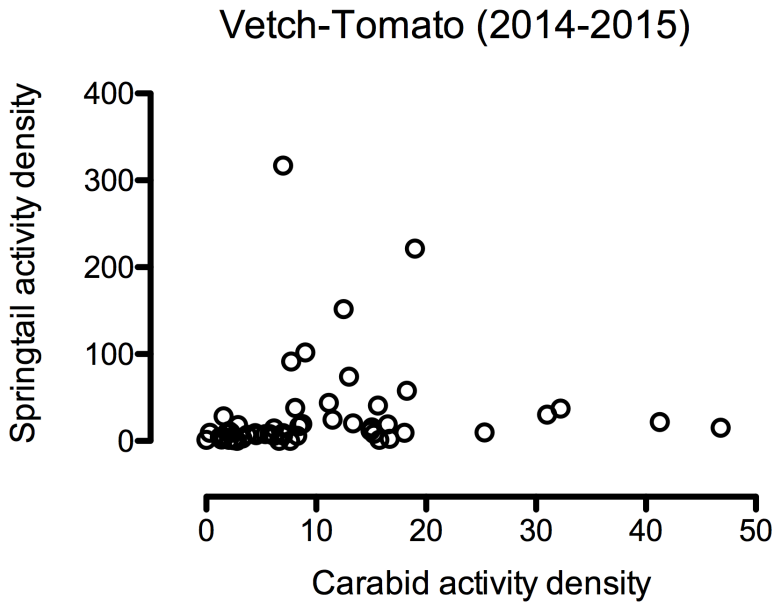

Figure 6 Correlation between carabid and springtail activity density pooling the two ASC termination techniques in experiment 1 (Spearman's rank correlation; R=0.5029, P< 0.0001).

Activity density of carabids and springtails in both years and for each ASC termination technique is reported in fig.7 and 8.

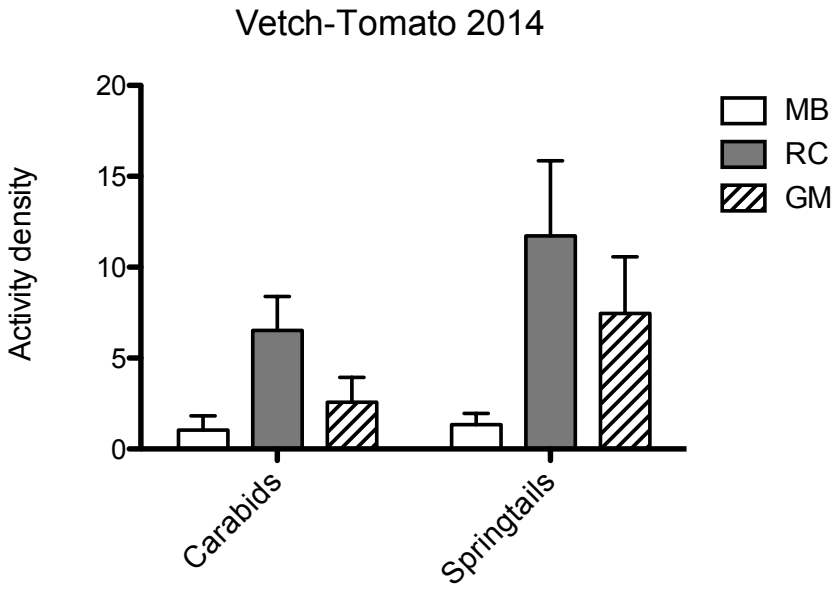

Figure 7 Carabid and springtail activity density in experiment 1 for 2014 (MB= synthetic biodegradable film cover, RC=roller crimper, GM=green manure). Bars represent SE of mean.

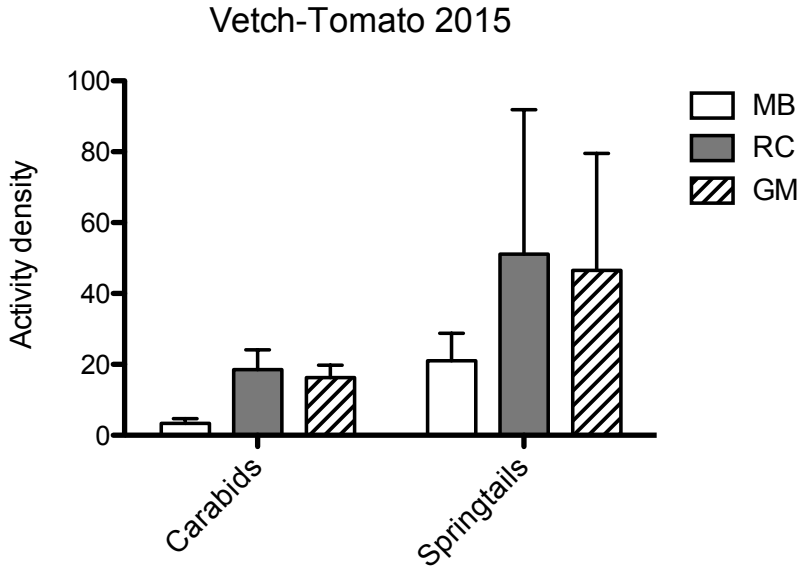

Figure 8 Carabid and springtail activity density in experiment 1 for 2015 (MB= synthetic biodegradable film cover, RC=roller crimper, GM=green manure). Bars represent SE of mean.

Furthermore, the activity density of carabids larger than 15 mm was positively correlated (fig.9) with the marks left on dummies by chewing insects (Spearman's rank correlation; R= 0.8093, P<0.0001).

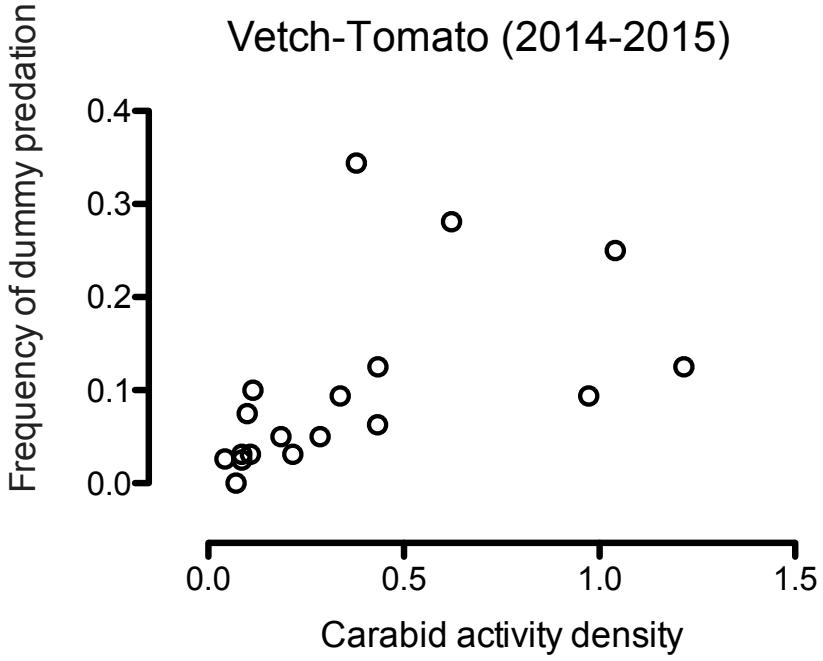

Figure 9 Correlation between carabid activity density and frequency of dummy predation in experiment 1 (Spearman's rank correlation; R= 0.8093, P<0.0001).

Pooling the two seasons (2014-2015), 1603 carabid individuals belonging to 42 species were collected (see appendix 1, 2 and 3 for details). The total number of carabids was higher in 2015 (N=1209) than in 2014 (N=394), corresponding to a mean activity density of 12.71 and 3.52 respectively. In total, we recorded more species in RC and GM than MB (Appendix 3). Moreover, 91.95% of the total was represented by four dominant species *Poecilus cupreus* (Linne), *Pseudophonus rufipes* (De Geer), *Brachinus sclopeta* (Fabricius) and *Harpalus distinguendus* (Duftschmid)). Larvae of *B.scopleta* are ectoparasites, developing on larvae of species such as *Harpalus* Latreille and *Amara* Bonelli. For this reason, the presence of *B.scopleta* is strictly related with the abundance of their hosts

(Burgio et al., 2014). The relative abundance of each species was reported in appendix 1; while carabid diet, wing dimorphism and body size can be found in appendix 2.

Experiment 2 (barley-zucchini)

Frequency of predation did not show any significant interactions with all the design variables tested (tab. 3).

Table 3 Results of the Log linear analysis, showing the interactions between the design variables (treatment-sampling date-year) and the response variables (predation) in experiment 2 (barley-zucchini).

Effect	**df**	**$\chi 2$**	**P**
Treatment*Predation	2	0.79	>0.05
Year*Predation	1	0.62	>0.05
Sampling date*Predation	4	2.57	>0.05
Treatment*Sampling Date*Predation	8	5.82	>0.05
Year*Treatment*Predation	2	1.10	>0.05
Year*Sampling Date*Predation	4	6.77	>0.05

The frequencies of attacked dummies for each treatment and year were reported in fig.10 and fig.11, respectively. Laboratory observation by stereomicroscope showed that chewing insects were responsible of most of the marks left on dummies (fig.12).

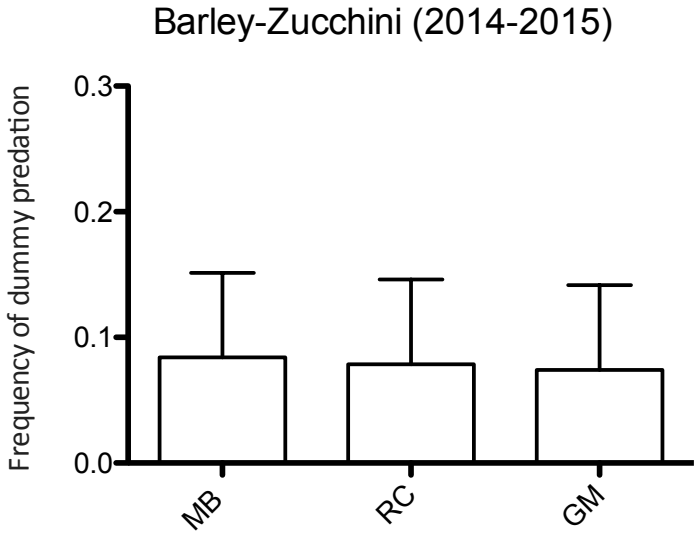

Figure 10 Frequency of dummy predation in the experiment 2 (barley-zucchini) per each treatment (MB=synthetic biodegradable film covering, RC=roller crimper, GM=green manure), pooling the seasons (2014-2015). Bars represent SE of binomial distribution.

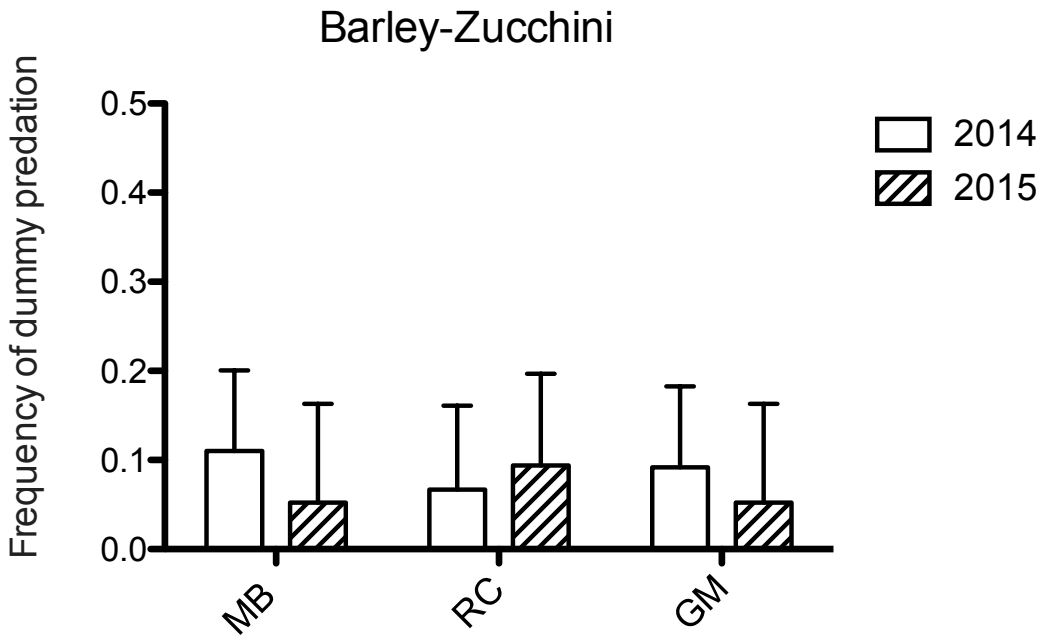

Figure 11 Frequency of dummy predation in experiment 2 (barley-zucchini) for each year (MB=synthetic biodegradable film covering, RC=roller crimper, GM=green manure). Bars represent SE of binomial distribution.

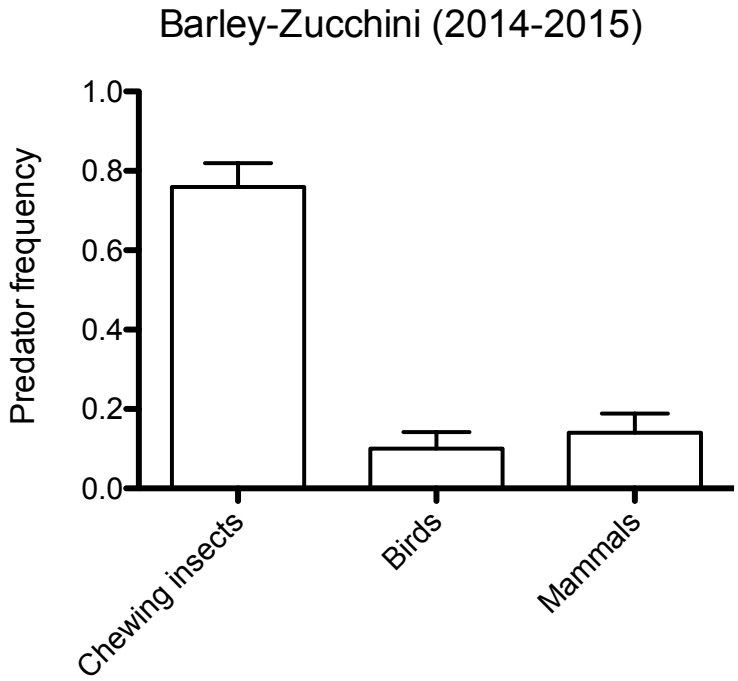

Figure 12 Distribution of the predation among the groups responsible for the marks left on dummies in experiment 2 (barley-zucchini) during both years (2014-2015). Bars represent SE of binomial distribution.

Activity density of carabids (tab. 4) and species richness (appendix 4) were higher in both years in ASC plots (tab 4, fig.13 and 14) in comparison with MB treatment, in both season. In this experiment neither significant correlation was detected between the activity density of carabids and that of springtails (Spearman's rank correlation; R=0.2584, P>0.05 (fig.15)) or between the activity density of carabids with size higher than 15 mm and the frequency of attacked dummies by chewing insects (Spearman's rank correlation; R= 0.1331, P= n.s (fig.16)).

Pooling the years a total of 1245 carabids and 27 species were collected. A higher number of individuals was sampled in 2015 (N=988) than in 2014 (N=257).

Pterostichus rufipes, *P. cupreus*, *Pterostichus melas* (Creutzer) were the most abundant species accounting for 94.70% of the total individual sampled.

The relative abundance of each species was reported in appendix 4; while carabid diet, wing dimorphism and body size can be found in appendix 2 and 3.

Table 4 Mean arthropod activity density (SE) sampled in experiment 2 (Barley-Zucchini) during 2014 and 2015; MB=synthetic biodegradable film covering, RC=roller crimper, GM=green manure.

Year	Treatments	Carabids	Springtails	Spiders	Rove beetles
2014	MB	2.04 (1.14)	2.47 (0.51)	1.64 (0.45)	0.38 (0.14)
	RC	3.46 (5.68)	3.51 (1.59)	7.83(4.13)	1.37 (0.81)
	GM	2.95 (2.79)	3.49 (1.42)	4.10 (1.06)	1.61 (0.60)
2015	MB	4.89 (2.25)	29.89 (15.15)	5.74 (1.81)	1.45 (0.89)
	RC	14.79 (9.31)	22.95 (23.18)	15.58 (5.12)	5.93 (2.16)
	GM	11.41 (6.24)	37.90 (34.17)	13.24 (4.82)	7.60 (3.79)

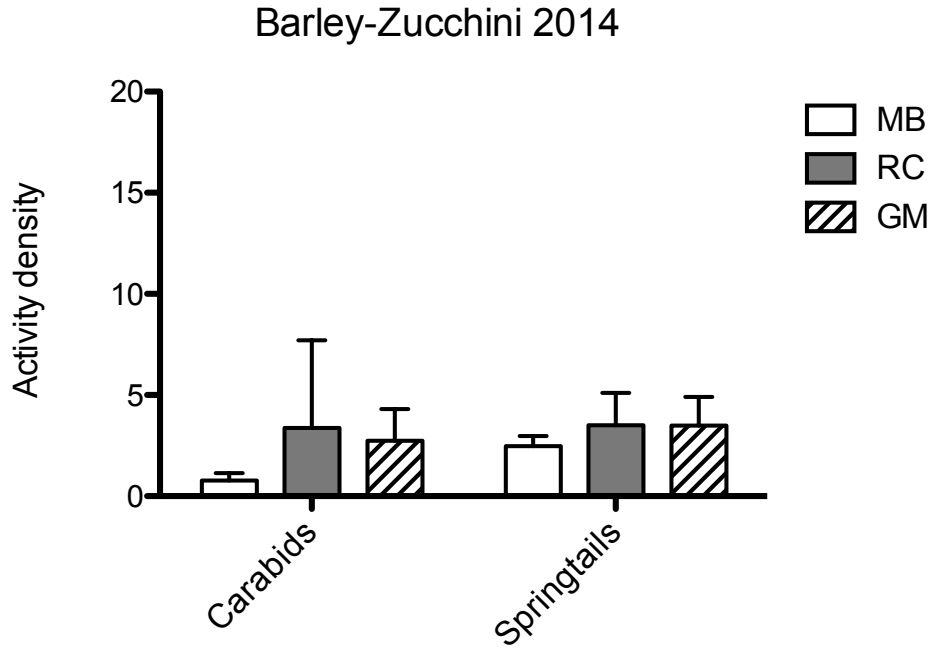

Figure 13 Carabid and springtail activity density during 2014 (Barley-Zucchini) per each treatment (MB= synthetic biodegradable film cover, RC=roller crimper, GM=green manure), in experiment 2. Bars represent SE of mean.

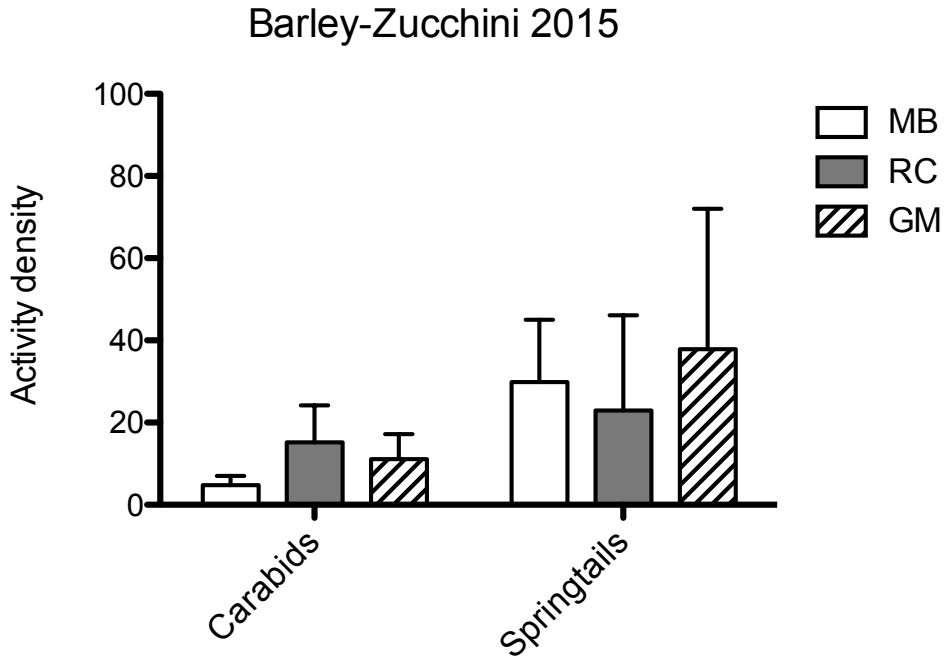

Figure 14 Carabid and springtail activity density during 2015 (Barley-Zucchini) per each treatment (MB= synthetic biodegradable film cover, RC=roller crimper, GM=green manure), in experiment 2. Bars represent SE of mean.

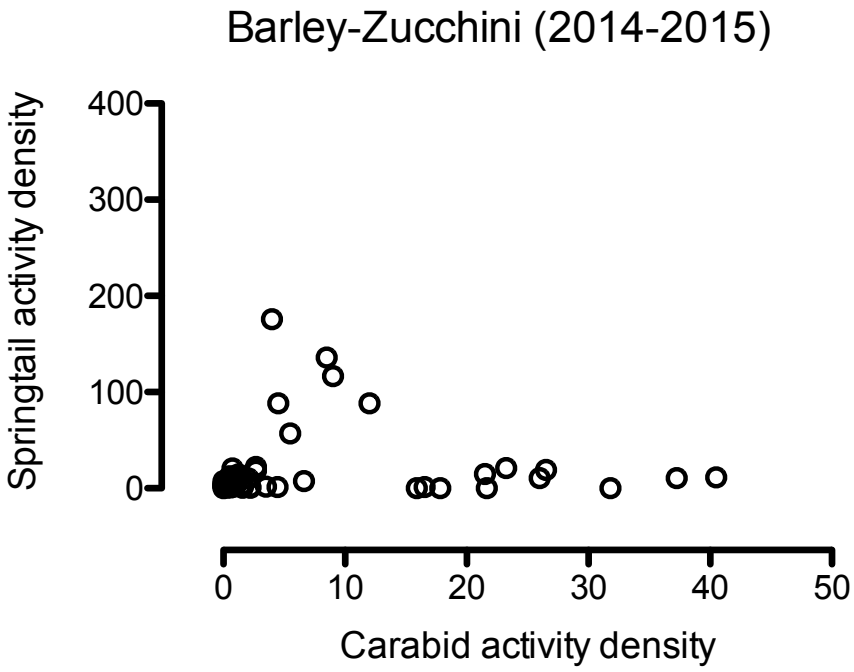

Figure 15 Correlation between carabid and springtail activity density pooling the two ASC termination techniques in experiment 2 (Spearman's rank correlation: R=0.2584 P>0.05).

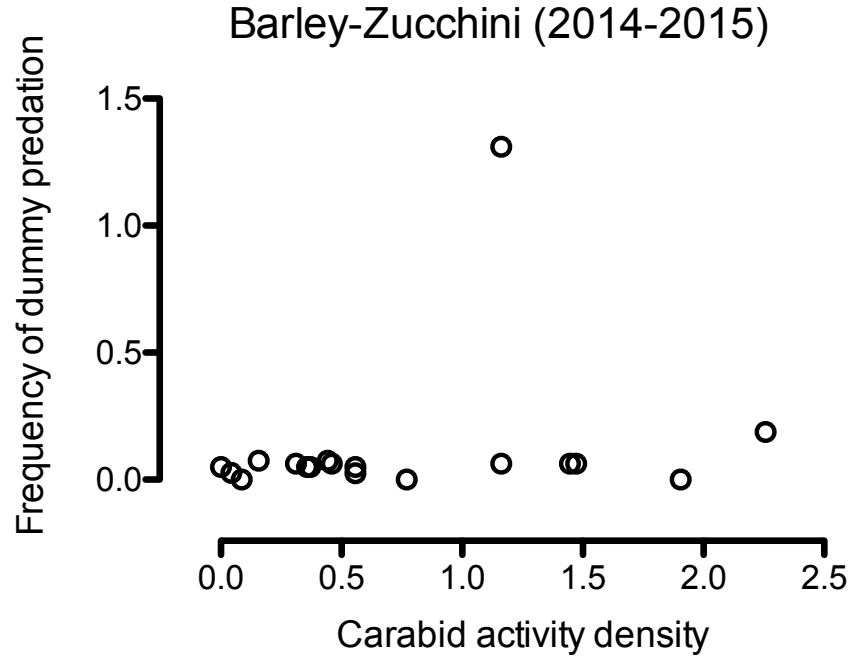

Figure 16 Correlation between carabid activity density and frequency of dummy predation in experiment 2 (Spearman's rank correlation; Barley-Zucchini, R=0.1331, P> 0.05).

3.5. Discussion and conclusion

Dummy caterpillars method was effective in estimating the predation rate in the present experiment. In particular, in our study the method was sensible in detecting differences in the intensity of ecosystem services among the tested cultivation systems. Dummy predation was higher in the conservation techniques (RC and GM) than MB system, but only the cover crop management based on vetch and RC resulted in a higher predation in comparison than GM. Also the significant correlation between the frequency of predation of dummy caterpillars and the activity density of carabids (> of 15 mm) in the vetch system (experiment 1) follows the same trend. Furthermore, the higher activity density of carabids in RC in comparison with GM in vetch system seems to demonstrate that the ecological impact of termination techniques can be affected by the cover crop used in the rotation (tab.2, fig.7 and 8). Natural enemies populations and arthropod diversity can be strongly affected by ASC used in the rotation, and cultivation technique and management (Pfiffner and Luka, 2003; Burgio et al., 2015; Depalo et al., 2016). In most cases, biodiversity was higher in organic management in comparison with conventional management, but the differences were strongly affected by the crop system. In our experiment, the rate of ASC decomposition was assumed to be quicker in vetch (experiment 1) than in barley (experiment 2). This result could be inferred taking into account the biotic processes occurred during the nitrogen immobilization, as resulted from literature data; in particular, ASCs with a low C/N ratio, such as leguminous plants, may directly affect the microbial community due to their higher nitrogen request (Kuo and Sainju, 1998; Hartwig and Ammon, 2002; Tosti et al., 2014). Moreover, microbial activity could be also enhanced by springtails (Lussenhop, 1992) through their grazing behaviour (Kaneda and Kaneko, 2011). Previous study has demonstrated (Gatiboni et al., 2011) that an increase of springtail populations occurred during vetch decomposition, confirming our

findings. Author's hypothesis focuses on the food habits of mesofauna, included springtails. Likely, fungi and bacteria are responsible of the fast rate of vetch decomposition and this could be the reason for the high density of springtails in relation to their feeding habit (Gatiboni et al., 2011; Kaneda and Kaneko, 2011). Many generalist predators prey on springtails and this evidence highlights the importance of this group in the trophic chains. We supposed that generalist predators like carabids can be supported by feeding on soil organisms such as springtails, whose abundance was enhanced by high level of nitrogen immobilization in vetch.

Besides the trophic-web interpretation, vetch could have also determined a lower disturbance of soil in comparison with barley, leading a higher dummy predation, though a direct comparison between the two different experiments cannot be done. Results of our experiments, corroborated by other studies, seem to confirm that vetch, as cover crop, is responsible of an increase of ecosystem services, including predation, in our studied system.

In conclusion, the ecological sustainability of ASC terminations (roller crimper and green manure) was higher in the experiment with vetch, in comparison with barley. In this context, predation pressure assessed by dummy caterpillars proved to be a sensitive method to assess the predation activity and, in an indirect way, the sustainability and conversely, the disturbance of soil conservation techniques. This study demonstrated a strong interaction between ASC used in the rotation with the termination techniques and the cash crops cultivated. Considering the importance of soil predation in conservation biological control, further studies should be done in order to investigate the influence of the soil management on ecosystem services. In this context, dummy caterpillar seems to be a practical and suitable method to measure ground-level predation. Flow chart reported at

the end of chapter 4 summarizes the main results obtained by ORTOSUP project (chapter 3 and 4 of my thesis).

3.6. Acknowledgement

This study was funded by the Organic Farming office of Ministero delle Politiche Agricole Alimentari e Forestali within the project ORTOSUP (Gestione agro-ecologica per la difesa delle colture orticole in biologico). We thank Dr.ssa L. Manici for project coordination, and M. Ferrante for advice and support with the dummy caterpillars method.

Appendix 1 Carabid species sampled in 2014-2015 in the experiment 1 (Vetch-Tomato) in relation to the two ASC terminations (RC=roller crimper, GM=green manure) and MB (biodegradable film covering). For each species we reported the number of individuals with their relative abundance (in the brackets).

	2014			2015			
Species (Vetch-Tomato)	**MB**	**RC**	**GM**	**MB**	**RC**	**GM**	**TOTAL**
Poecilus cupreus (L. 1758)	14 (3.55)	85 (21.57)	64 (16.24)	37 (3.06)	188 (15.55)	225 (18.61)	**613**
Pseudophonus rufipes (De Geer 1774)	15(3.81)	46 (11.68)	28 (7.11)	50 (4.14)	135 (11.17)	175 (14.47)	**449**
Brachinus (Brachynidius) sclopeta (F. 1792)		23 (5.84)	3 (0.76)	4 (0.33)	221 (18.28)	56 (4.63)	**307**
Harpalus distinguendus (Duftschmid 1812)	58 (1.27)	66 (16.75)	8 (2.03)	5 (0.41)	5 (0.41)	16 (1.32)	**105**
Pterostichus (Feronidius) melas (Creutzer 1799)	2 (0.51)	6 (1.52)	2 (0.51)	3 (0.25)	8 (0.66)	8 (0.66)	**29**
Amara aenea (De Geer 1774)		6 (1.52)			6 (0.50)	4 (0.33)	**16**
Bembidion quadrimaculatum (L. 1761)						12 (0.99)	**12**
Anisodactylus binotatus (F.1787)				2 (0.17)	7 (0.58)	2 (0.17)	**11**
Anchomenus dorsalis (Pontoppidan 1763)	2 (0.51)	2 (0.51)			1 (0.08)		**5**
Stenolophus teutonus (Schrank 1781)		1 (0.25)			3 (0.25)	1 (0.08)	**5**
Stenolophus proximus Dejean 1829						4 (0.40)	**4**
Trechus quadristriatus (Schrank 1781)	1 (0.25)				3 (0.25)		**4**
Harpalus oblitus Dejean 1829		1 (0.25)	1 (0.25)	1 (0.08)			**3**
Parophonus maculicornis (Duftschmid 1812)					3 (0.25)		**3**
Parophonus planicollis (Dejean 1829)			1 (0.25)		2 (0.17)		**3**

	2014			2015			
Species (Vetch-Tomato)	**MB**	**RC**	**GM**	**MB**	**RC**	**GM**	**TOTAL**
Ocydromus (Peryphanes) latinus (Netolitzky 1911)	1 (0.25)	1 (0.25)					**2**
Dixus clypeatus (P. Rossi 1790)	2 (0.51)						**2**
Amara eurynota (Panzer 1796)					1 (0.08)	1 (0.08)	**2**
Chlaeniellus nitidulus (Schrank 1781)					1 (0.08)	1 (0.08)	**2**
Microlestes corticalis (L. Dufour 1820)						2 (0.17)	**2**
Polyderis algiricus (Lucas 1846)				1 (0.08)		1 (0.08)	**2**
Calathus (Bedelinus) circumseptus Germar 1824		1 (0.25)					**1**
Calathus (Neocalathus) erratus (Sahlberg 1827)		1 (0.25)					**1**
Carabus (Procrustes) coriaceus (L. 1758)		1 (0.25)					**1**
Harpalus dimidiatus (P. Rossi 1790)		1 (0.25)					**1**
Ophonus (Hesperophonus) azureus (F. 1775)		1 (0.25)					**1**
Amara lunicollis Schiodte 1837	1 (0.25)						**1**
Philochthus lunulatus (Geffroy in Fourcroy 1785)	1 (0.25)						**1**
Chlaeniellus olivieri Crotch 1871					1 (0.08)		**1**
Chlaenius (Trichochlaenius) chrysocephalus (P. Rossi 1790)					1 (0.08)		**1**
Parophonus mendax (Rossi 1790)					1 (0.08)		**1**
Bembidion ambigua (Dejean, 1831)						1 (0.08)	**1**

	2014			2015			
Species (Vetch-Tomato)	**MB**	**RC**	**GM**	**MB**	**RC**	**GM**	**TOTAL**
Acupalpus paludicola (Reitter 1884)						1 (0.08)	**1**
Bembidion lampros (Herbst. 1784)						1 (0.08)	**1**
Calathus (Amphyginus) rotundicollis (Dejean 1828)						1 (0.08)	**1**
Chlaenius (Dinodes) viridis Menetries. 1832				1 (0.08)			**1**
Cicindela campestris (L. 1758)						1 (0.08)	**1**
Dolichus halensis (Schaller 1783)				1 (0.08)			**1**
Nebria brevicollis (F. 1792)						1 (0.08)	**1**
Pseudophonus griseus (Panzer. 1797)				1 (0.08)			**1**
Pterostichus (Adelosia) macer (Marsham 1802)						1 (0.08)	**1**
Pterostichus (Argutor) vernalis (Panzer 1796)						1 (0.08)	**1**
Indeterminate		1 (0.25)					**1**
TOTAL	**44**	**243**	**107**	**106**	**587**	**516**	**1603**

Appendix 2 Carabid species found in both years with diet, wing dimorphism and body size

Species	Experiment	Diet	Wing dimorphism	Body size
Acupalpus paludicola	Vetch-Tomato	Ph/Zo	Ma	3-4
Amara aenea	Barley-Zucchini	Ph	Ma	6-8
Amara eurynota	Vetch-Tomato	Ph	Ma	9-13
Amara lunicollis	Vetch-Tomato	Ph	Ma	6-9
Anchomenus dorsalis	Vetch-Tomato	Zo	Ma	5-8
Anisodactylus binotatus	Barley-Zucchini & Vetch-Tomato	Ph	Ma	9-12
Bembidion lampros	Vetch-Tomato	Zo	Ma	2-4
Bembidion quadrimaculatum	Vetch-Tomato	Zo	Ma	2-3
Bembidion ambigua (Dejean 1831)	Vetch-Tomato	Zo	Ma	3-4
Bembidion Subg.Ocydromus	Barley-Zucchini	Zo		5-7
Brachinus (Brachynidius) sclopeta	Barley-Zucchini & Vetch-Tomato	Zo	Ma	4-7
Calathus (Amphyginus) rotundicollis	Vetch-Tomato	Zo	Di	8-11
Calathus (Bedelinus) circumseptus	Barley-Zucchini & Vetch-Tomato	Zo	Ma	10-14

Species	Experiment	Diet	Wingdimorphism	Bodysize
Calathus (Neocalathus) erratus	Vetch-Tomato	Zo	Ma	8-12
Calathus cinctus	Barley-Zucchini	Zo	Di	6-9
Calathus fuscipes	Barley-Zucchini	Zo	Di	10-14
Carabus (Procrustes) coriaceus	Vetch-Tomato	Zo	Br	32-42
Chlaeniellus nitidulus	Vetch-Tomato	Unknown	Ma	10-20
Chlaeniellus olivieri	Vetch-Tomato	Unknown	Ma	10-12
Chlaenius (Dinodes) decipiens	Barley-Zucchini	Unknown	Ma	10-13
Chlaenius (Dinodes) viridis Menetries, 1832	Vetch-Tomato	Unknown	Ma	10-11
Chlaenius (Trichochlaenius) chrysocephalus	Vetch-Tomato	Unknown	Ma	7-10
Cicindela campestris	Barley-Zucchini & Vetch-Tomato	Zo	Ma	12-15
Dixus clypeatus	Vetch-Tomato	Ph	Ma	7-13
Dolichus halensis	Vetch-Tomato	Zo	Ma	13-20
Elaphrus (Elaphroterus) aureus	Barley-Zucchini	Zo	Ma	5-7
Gynandromorphus etruscus	Barley-Zucchini	Unknown	Ma	10-11

Species	Experiment	Diet	Wingdimorphism	Bodysize
Harpalus dimidiatus	Barley-Zucchini & Vetch-Tomato	Ph	Ma	11-14
Harpalus distinguendus	Barley-Zucchini & Vetch-Tomato	Ph	Ma	7-11
Harpalus oblitus	Barley-Zucchini & Vetch-Tomato	Ph	Ma	8-11
Harpalus pygmaeus	Barley-Zucchini	Ph	Ma	5-7
Microlestes corticalis	Barley-Zucchini & Vetch-Tomato	Zo	Ma	2-3
Nebria brevicollis	Vetch-Tomato	Zo	Ma	9-14
Ocydromus (Peryphanes) latinus	Vetch-Tomato	Zo	Ma	5-6
Ophonus (Hesperophonus) azureus	Barley-Zucchini & Vetch-Tomato	Ph	Di	6-9
Ophonus stictus	Barley-Zucchini	Ph	Ma	11-17
Parophonus hispanus	Barley-Zucchini	Ph	Ma	8-11
Parophonus maculicornis	Vetch-Tomato	Ph	Ma	5-7
Parophonus mendax	Vetch-Tomato	Ph	Ma	6-10
Parophonus planicollis	Barley-Zucchini & Vetch-Tomato	Ph	Ma	6-10
Philochthus lunulatus	Vetch-Tomato	Zo	Ma	3-4

Species	Experiment	Diet	Wingdimorphism	Bodysize
Poecilus cupreus	Barley-Zucchini & Vetch-Tomato	Ph/Zo	Ma	9-13
Polyderis algiricus	Vetch-Tomato	Unknown	Br	1-2
Pseudophonus griseus	Barley-Zucchini & Vetch-Tomato	Ph/Zo	Ma	9-11
Pseudophonus rufipes	Barley-Zucchini & Vetch-Tomato	Phyt/Zo	Di	11-16
Pterostichus (Adelosia) macer	Vetch-Tomato	Zo	Ma	11-15
Pterostichus (Argutor) vernalis	Vetch-Tomato	Zo	Di	6-7
Pterostichus (Feronidius) melas	Barley-Zucchini & Vetch-Tomato	Zo	Br	14-18
Pterostichus (Platysma) niger	Barley-Zucchini	Zo	Di	15-22
Scybalicus oblongiusculus	Barley-Zucchini	Ph	Ma	10-13
Stenolophus proximus	Vetch-Tomato	Ph/Zo	Ma	6-7
Stenolophus teutonus	Vetch-Tomato	Ph/Zo	Ma	5-7
Trechus quadristriatus	Barley-Zucchini & Vetch-Tomato	Zo	Di	3-4

Appendix 3 Total numbers of carabid species for both years considering the two ASC termination techniques (RC=roller crimper and GM=green manure) and MB (biodegradable film covering). We also reported the number of species in relation to their diet.

Experiment	Treatment	Total number of carabid species	Number of zoophagous carabid species	Numbero of phytophagous carabid species	No available information about the diet
Vetch-Tomato	MB	**17**	10	5	2
Vetch-Tomato	RC	**25**	11	11	3
Vetch-Tomato	GM	**24**	16	6	2
Barley-Zucchini	MB	**13**	7	6	
Barley-Zucchini	RC	**16**	9	6	1
Barley-Zucchini	GM	**16**	9	6	1

Appendix 4 Carabid species sampled in 2014-2015 in the experiment 2 (Barley-Zucchini) in relation to the two ASC terminations (RC=roller crimper, GM=green manure) and MB (biodegradable film covering). For each species we reported the number of individuals with their relative abundance (in the brackets).

	2014			2015			
Species (Barley-Zucchini)	**MB**	**RC**	**GM**	**MB**	**RC**	**GM**	**TOTAL**
Pseudophonus rufipes	12 (4.67)	102 (39.69)	60 (23.35)	111 (11.23)	360 (36.44)	252 (25.51)	**897**
Poecilus cupreus		11 (4.28)	23 (8.95)	28 (2.83)	51 (5.16)	58 (5.87)	**171**
Pterostichus (Feronidius) melas	13 (5.06)	8 (3.11)	7 (2.72)	3 (0.30)	55 (5.57)	25 (2.53)	**111**
Harpalus distinguendus		1 (0.39)	7 (2.72)	4 (0.40)		1 (0.10)	**13**
Brachinus (Brachynidius) sclopeta				1 (0.10)	6 (0.61)	4 (0.40)	**11**
Trechus quadristriatus	1 (0.39)	2 (0.78)	2 (0.78)			4 (0.40)	**9**
Harpalus dimidiatus					1 (0.10)	3 (0.30)	**4**
Harpalus oblitus				1 (0.10)	2 (0.20)	1 (0.10)	**4**
Pterostichus (Platysma) niger (Schaller 1783)					2 (0.20)	2 (0.20)	**4**
Scybalicus oblongiusculus (Dejean 1829)	1 (0.39)	1 (0.39)		1 (0.10)			**3**
Anisodactylus binotatus					1 (0.10)	1 (0.10)	**2**
Elaphrus (Elaphroterus) aureus Muller 1821		1 (0.39)					**1**
Ophonus (Hesperophonus) azureus	1 (0.39)						**1**
Ophonus stictus Stephens 1828	1 (0.39)						**1**
Amara aenea				1 (0.10)			**1**

	2014			2015			
Species (Barley-Zucchini)	**MB**	**RC**	**GM**	**MB**	**RC**	**GM**	**TOTAL**
Bembidion Subg.Ocydromus Clairville 1806				1 (0.10)			**1**
Calathus cinctus Motschulsky 1850					1 (0.10)		**1**
Calathus (Bedelinus) circumseptus			1 (0.39)				**1**
Chlaenius (Dinodes) decipiens (Dufour. 1820)					1 (0.10)		**1**
Cicindela campestris					1 (0.10)		**1**
Gynandromorphus etruscus (Quensel in Schonherr 1806)			1 (0.39)				**1**
Calathus fuscipes (Goeze 1777)			1 (0.39)				**1**
Harpalus pygmaeus Dejean 1829						1 (0.10)	**1**
Microlestes corticalis						1 (0.10)	**1**
Parophonus hispanus (Rambur. 1838)					1 (0.10)		**1**
Parophonus planicollis						1 (0.10)	**1**
Pseudophonus griseus				1 (0.10)			**1**
TOTAL	**29**	**126**	**102**	**152**	**482**	**354**	**1245**

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4. Influence of agro-ecological service crop termination and synthetic biodegradable film covering on *Aphis gossypii* Glover (Rhynchota: Aphididae) infestation and natural enemy dynamics

4.1. Abstract

Agro-ecological service crops (ASC) can increase the vegetation complexity of agro-ecosystems leading to a positive impact on natural enemies of arthropod pests and on weed control. In this study, two ASC terminations (green manure and roller crimper) and a Mater-Bi-mulched control (MB) were compared in order to describe the effects on pests and beneficial dynamics in an organic vegetable system. The trials were conducted over two consecutive growing seasons in 2014 and 2015. Zucchini were grown as cash crop and barley as ASC. Pests and natural enemies were monitored fortnightly by visual samplings along the whole zucchini-growing season. Zucchini plants showed a faster vegetative growth in Mater-Bi treatment than in ASC terminations. In both years, MB plots were characterized by higher soil temperature and higher leaf N concentration resulting in plants more susceptible to *Aphis gossypii* infestations. In all the experimental plots, natural enemies controlled aphid infestations and no insecticide and sprays were necessary.

In conclusion, the tested ASC techniques have been suggested as a tool to mitigate aphid infestation.

Keywords: cover crops, barley, no-till, natural enemies, roller crimper, zucchini.

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4.2. Introduction

Conventional agriculture strongly impacts the environment. Massive use of pesticides, herbicides and fertilizers is responsible of air, water and soil pollution (Tilman et al., 2002; Foley et al., 2011) and the reduction of agricultural inputs is a primary goal of sustainable agriculture (Tilman et al., 2002). Since 1958, Elton theorized and argued that simplified agro-ecosystems are more vulnerable to pest insect infestations than complex communities. Agricultural intensification, including monoculture, intense use of inputs, conventional tillage and low species diversity can lead to outbreaks of pests that cause heavy damage to crops (Hooks et al., 1998; Altieri, 1999; Altieri and Nicholls, 2004;). Non-renewable agricultural inputs should be reduced to preserve ecosystems and biodiversity in order to promote sustainable agriculture (Horrigan et al., 2002).

A basic contribution in this direction has been obtained by means of habitat manipulation, including the introduction in the rotation of crops not aimed to yield rather to provide agroecological services (Altieri, 1999; Bianchi et al., 2013). Different terms have been used so far to indicate the crops having an environmental and or an agronomic role (i.e. catch crops, cover crops, complementary crops and green manure). However, these names were often connected with a specific purpose or with a specific position in the rotation and they have not always been considered completely appropriate in any situation or broad enough to encompass all the crops having agro-environmental functions. Therefore, the new terminology of *Agro-ecological Service Crops* (ASC) was recently introduced to overcome this issue (Canali et al., 2015).

ASC may provide important benefits to natural enemies, by increasing the structural and vegetation complexity within agro-ecosystems (Johnson et al., 2004; Bryant et al., 2013;). Beside the selection of the plant species complex, the potential benefits of ASC may be influenced by several factors, including method and timing of termination. Among the

available techniques to kill ASC, no-till roller crimper approach was suggested as an effective solution to control weeds, reduce soil erosion and diminish fuel and energy consumption (Ashford and Reeves, 2003; Altieri et al., 2011; Luna et al., 2012). The main agronomic questions related to this technique are: (a) the right optimum of ASC biomass before rolling, (b) the nitrogen immobilization and (c) the re-growth of ASC during the subsequent cash crop cycle. Canali et al. (2013) proposed for vegetable crops an innovative roller crimper that flattens the ASC while simultaneously creating a transplanting furrow (in line till/roller crimper).

In spite of some promising results that demonstrate the positive effects of cover crops on natural enemies (Carmona and Landis, 1999; Schmidt et al., 2004; Pullaro et al., 2006; Larentzaki et al., 2008; Rodríguez et al., 2012), the role of ASC on insect dynamics has not yet been tested exhaustively. A better knowledge of the relations among "ASCs-agronomic techniques-arthropod fauna" might lead to a strategic use ASC for pest management and beneficial insect conservation.

The objective of this study was to evaluate the impact of two different ASC terminations (green manuring *vs* in line till/roller crimping) on pest and beneficial dynamics, in an organic vegetable system. The new roller crimper technology (Canali et al., 2013) was compared with the green manure technique for ASC management. A synthetic biodegradable film cover, which is the conventional mulching technique used in many vegetable crops, was used as control. Concerning pest dynamics, particular attention was given to the melon or cotton aphid, *Aphis gossypii* Glover (Rhynchota: Aphididae), which is considered the key pest on zucchini (*Cucurbita pepo* L.). The plant was selected as cash crop in this study because its economic importance. This aphid causes damages on leaves, transmitting viruses and reducing the commercial value of fruits as a result of honeydew

production (Ebert and Cartwright, 1997). For this reason, trophic webs related to *A. gossypii* were monitored, including predator and parasitoid species.

4.3. Materials And Methods

4.3.1.1. Site, experiment and treatments

Field experiments were conducted at CREA Horticulture Research Unit of Monsampolo del Tronto (Central Italy) in an organic zucchini field (21x24 m). The tested treatments were: i. in line till/roller crimper (RC) and ii. green manure (GM). The two ASC termination techniques were compared to synthetic biodegradable film covering (Mater-Bi, MB), a common technique used to control weeds in Italy in many vegetable systems. Three plots per treatment were replicated and the whole experiment design was repeated for two consecutive seasons (2014 and 2015), by changing the spatial distribution of the treatments in the zucchini field.

The ASC crop, barley (*Hordeum vulgare* L.), was sown on October 31th 2013 and on October 21th 2014 with a sown density of 200 kg/ha in both years. Zucchini plants (*cultivar* "Zuboda") were transplanted after ASC termination techniques, for a total of 0.83 plants/m^2. This *cultivar*, supplied by Arcoiris srl (Modena, Italy) was selected for the study because it lacks resistance genes against the main biotic stresses. Transplanting was carried out on May 7th 2014 and on May 6th 2015. No insecticides and herbicides were used during the two growing seasons. The fertilization was the same in each treatment during the two years: N (50 kg/ha), P_2O_5 (13 kg/ha) and K_2O (21 kg/ha).

4.3.1.2. Yield assessment

In 2014, the harvest started on June 10th and ended on August 13th, the whole cropping cycle lasting 64 days. In 2015, the harvest started on June 5th and ended on August 3rd, the whole cropping cycle lasted 59 days. The harvest was made according to fruit ripening by

collecting fruits three times per week along the whole harvest period. Zucchini yield was evaluated according to the local market standards within 24 h from the harvest.

4.3.1.3. *Pest and beneficial insect sampling*

Pest and beneficial insects were monitored fortnightly, from June till the beginning of August, by visual sampling. In each plot, ten to fifteen plants were sampled by observing three randomly selected leaves for each plant. In particular, ten plants per plot were sampled during the first and last sampling date in 2014 season; the same sampling size was used during the first date in 2015 season.

A.gossypii was the predominant pest collected by samplings. This species is the key pest on cucurbits in open and protected crops in Mediterranean countries (Ebert and Cartwright, 1997). Aphid colonies were ranked by visual sampling into four different infestation classes: 0 (no aphids), 1 (few scattered aphids), 2 (small aphid colonies) and 3 (extended colonies or leaf surface almost covered by aphids).

All the beneficial insects found on the leaves, including parasitoid mummies, were counted during the visual sampling.

4.3.1.4. Chlorophyll measurement

The quantity of chlorophyll was analysed twice a years at 68 and 85 days after transplanting (DAT) in 2014, and at 55 and 65 DAT in 2015. Data were obtained with a hand held chlorophyll meter (CCM 200, Opti-Sciences, Tyngsboro, Massachusetts, USA). This instrument allows calculating the chlorophyll content index (CCI) based on absorbance measurements at 653 and 931 nm. The claimed accuracy of the CCM-200 is ±1.0 CCI units. Three leaves from nine randomly selected plants were analysed from each plot. The measure was repeated twice on each leaf. The CCI results were utilized to

calculate the Soil and Plant Analysis Development (SPAD) index, an indirect measure of nitrogen within plant tissues.

4.3.1.5. Soil temperature measurement

HD 207-1 data loggers (Delta Ohm, Caselle di Selvazzano, Padua, Italy) were used to measure soil temperature (0-10 cm depth). Data were recorded every 30 minutes from 14th of May to 13th of August in 2014 and from 5th of June to 7thof August in 2015. Data were analysed by DeltaLog2 dedicated software.

4.3.1.6. *Data analysis*

Aphid infestation was evaluated using the Townsend-Heuberger's formula:

$$P(\%\ of\ infestation) = \frac{\sum_v (N_v \times v)}{(n-1) \times N_T} \times 100$$

where:

n=Number of infestation classes

N_v=Number of leaves in each class of infestation

v=Value of the different classes of infestation (from 0 to 3)

N_T=Total number of sampled leaves

Mean number of natural enemies per leaf was also calculated for each sampling date.

The correlation between natural enemies (predators and parasitoids), and infestations of *A. gossypii* were analysed by Spearman's rank method.

Log linear analysis (Haberman et al., 1976) was used to test the interaction between design variables (treatment-sampling date-year) and response variables (weighted aphid infestation), by a multi-way contingency table. The obtained matrix was based on the frequency of leaves belonging to the four infestation classes. By means of log linear analysis, only the associations involving the response variable were selected.

The significant interactions revealed by log linear analysis were further analysed with a chi-square test for independence; this test was used to understand any association between different categorical variables with more than two levels (Beasley and Schumacker, 1995; Garcia-Perez and Núñez-Antón, 2003; Sharpe D, 2015).

Data analyses were performed using Statistica ver. 7.0 (Statsoft Italia) and SPSS ver.13.0 (Statistical Package for Social Science, USA).

4.4. Results

Log linear analysis (tab. 1) showed significant interactions of aphid infestation with: i. year (P<0.001), ii. sampling dates (P<0.001) and iii. treatment (P<0.001). Infestation of *A .gossypii* (fig.1) was higher in 2014 than in 2015, in all the treatments (significant interaction "treatment*infestation", chi-square= 65.66; d.f.=6; P<0.001).

Table 1 Results of the Log linear analysis that show the interactions between the design variables (treatment-sampling date-year) and the response variables (aphid infestation).

Effect	df	$\chi 2$	P
Year*Sampling date	3	20.383	<0.001
Year*Treatment	2	~0	>0.05
Year*Infestation	3	191.712	<0.001
Sampling date*Treatment	6	~0	<0.001
Sampling date*Infestation	9	851.661	<0.001
Treatment*Infestation	6	115.537	<0.001
Year*Sampling date*Treatment	6	~0	>0.05
Year*sampling date*Infestation	9	151.591	<0.001
Year*treatment*Infestation	6	49.837	<0.001
Sampling date*Treatment*Infestation	18	85.612	<0.001

In MB treatment, higher infestations of *A. gossypii* were detected in comparison with those observed in the treatments based on ASC (fig 1; tab.1); it is remarkable that this trend was observed in both seasons.

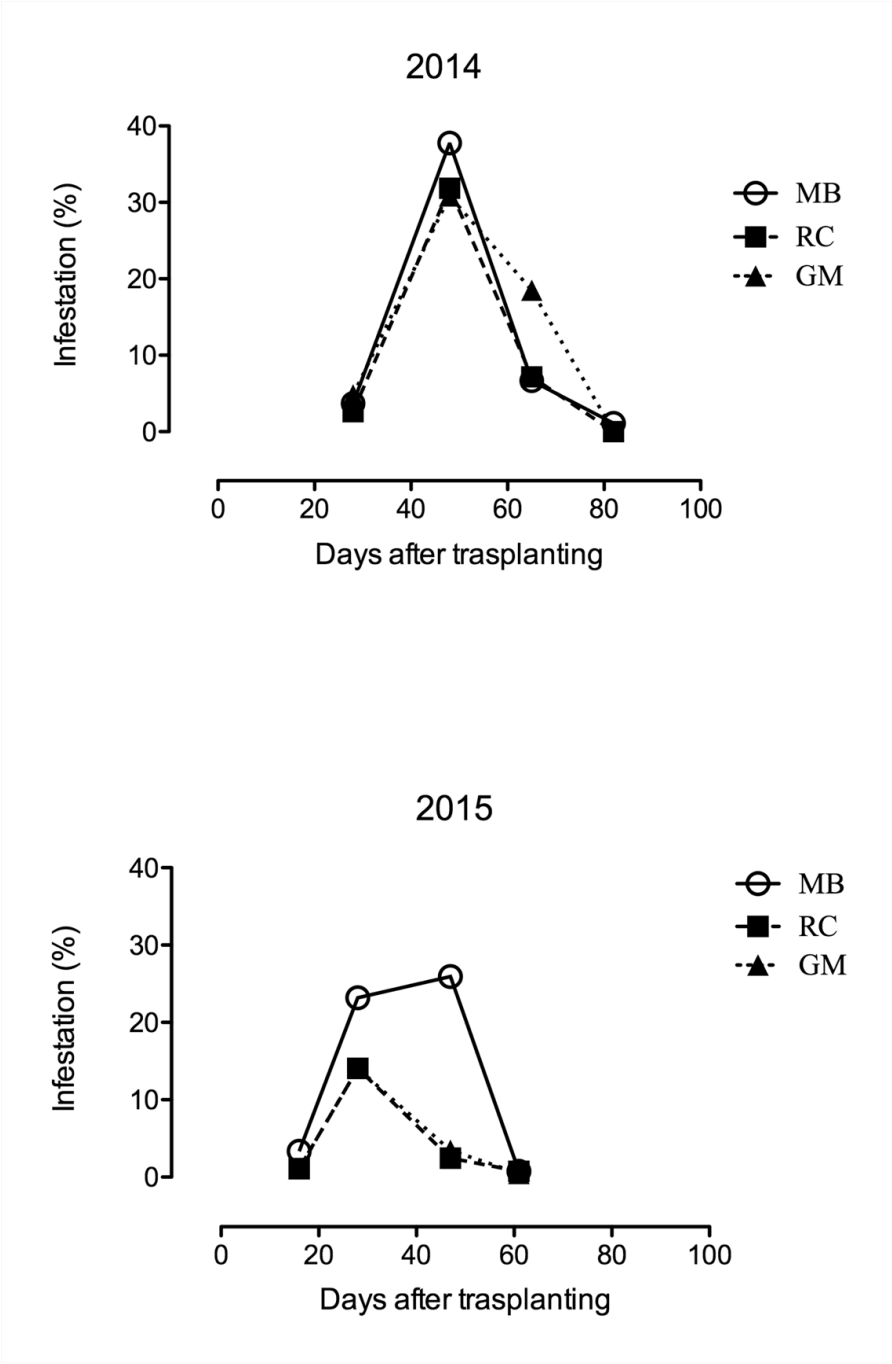


Figure 1 Trends of aphid infestations (expressed as weighted percentage (%) in each treatment during the two sampling seasons. MB=synthetic biodegradable film covering, RC=roller crimper, GM=green manure.

Heavily infested leaves belonging to class 2 and 3 were found only in MB treatment; the only exception was observed in GM during the second sampling date in 2015 (28 DAT), when only one leaf was scored as "class 2" of infestation (fig.2). In MB plots, class 3 of infestations were recorded in 2014 at 48 DAT (37.78%) and in 2015 at 47 DAT (25.93%), causing the significant interaction among "sampling date*treatment*infestation" (chi-square= 85.6; d.f.=18; P<0.001).

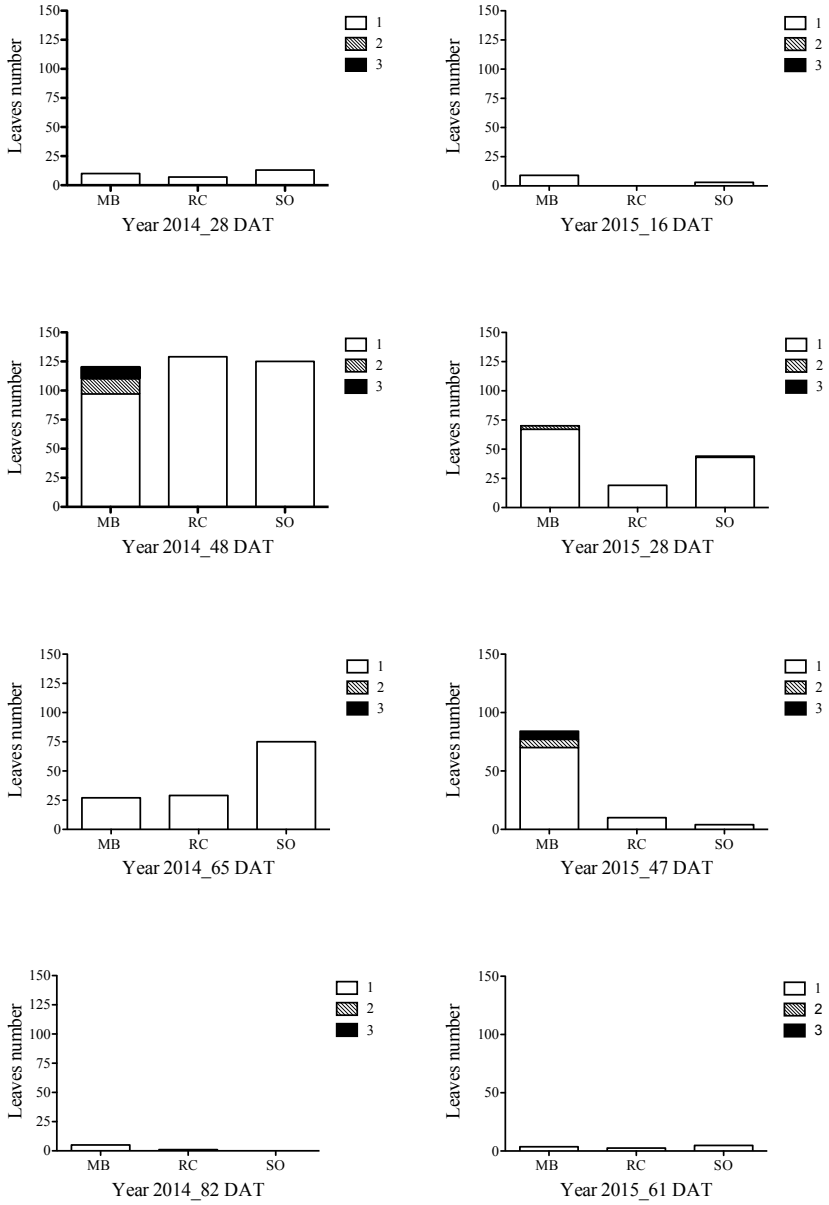


Figure 2 Number of leaves scored into the different classes of aphid infestation (class1, 2 and 3) during 2014, in each treatment and sampling date. MB=synthetic biodegradable film covering, RC=roller crimper, GM=green manure.

Aphid occurrence and peak of infestation were recorded earlier in 2015 than in 2014, in all the treatments (significant interaction "year*sampling date*infestation", chi-square=151.5, d.f.=9, P<0.001). Also, temporal pattern of infestation in MB was different in comparison with that of GM and RC (explained by the significant "year*treatment*infestation" interaction, chi-square=49.8, d.f.=6, P<0.001).

RC resulted in lower SPAD index in all surveys, with the exception of the values obtained at 85 DAT in GM (fig.3).

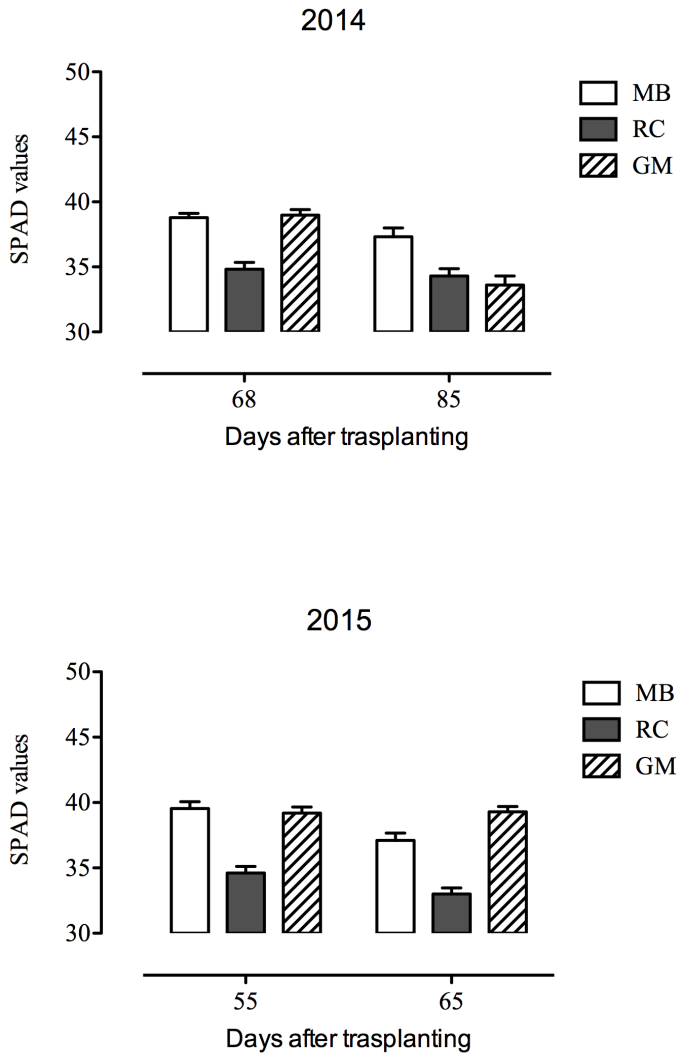


Figure 3 SPAD (Soil and Plant Analyzer Development index) values during 2014-2015, an indirect measure of nitrogen within plant tissues. MB=synthetic biodegradable film covering, RC=roller crimper, GM=green manure. Bars represent SE of mean.

In both years, soil temperature was higher in MB plots in comparison with the other treatments (fig.4); GM plots registered intermediate temperature values between MB and RC, although they were close to MB in 2015. The average soil temperature of RC plots was 21.7-22.9 °C (2014-15), while those registered in GM and MB were 22.5-25.1 °C and 24.1-25.8 °C.

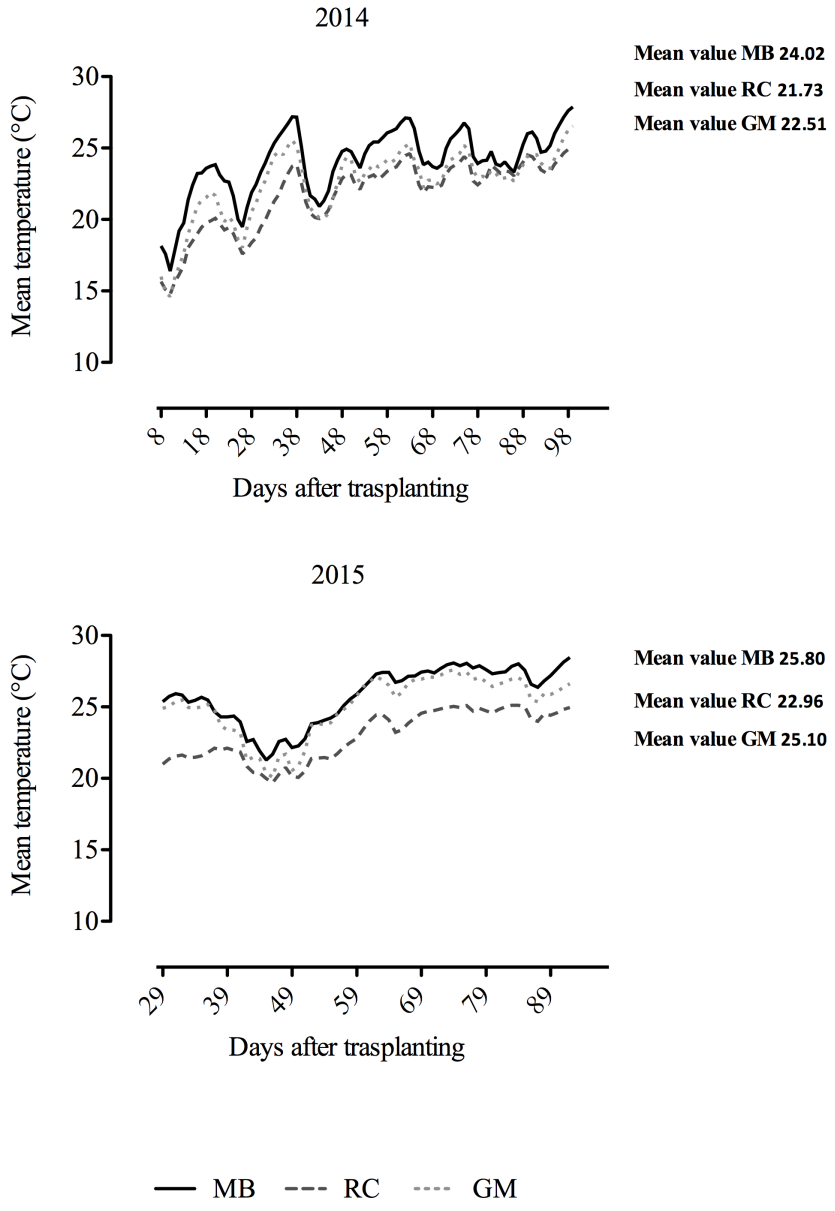


Figure 4 Soil temperature (mean daily value) in both sampling season. MB=synthetic biodegradable film covering, RC=roller crimper, GM=green manure

.

Responses of predators and parasitoids were density-dependent and their abundance was positively correlated with *A.gossypii* infestations (Spearman's rank correlation; R= 0.61, P<0.01 and R= 0.44, P<0.05, for predators (a) and parasitoids (b) respectively (fig. 5)).

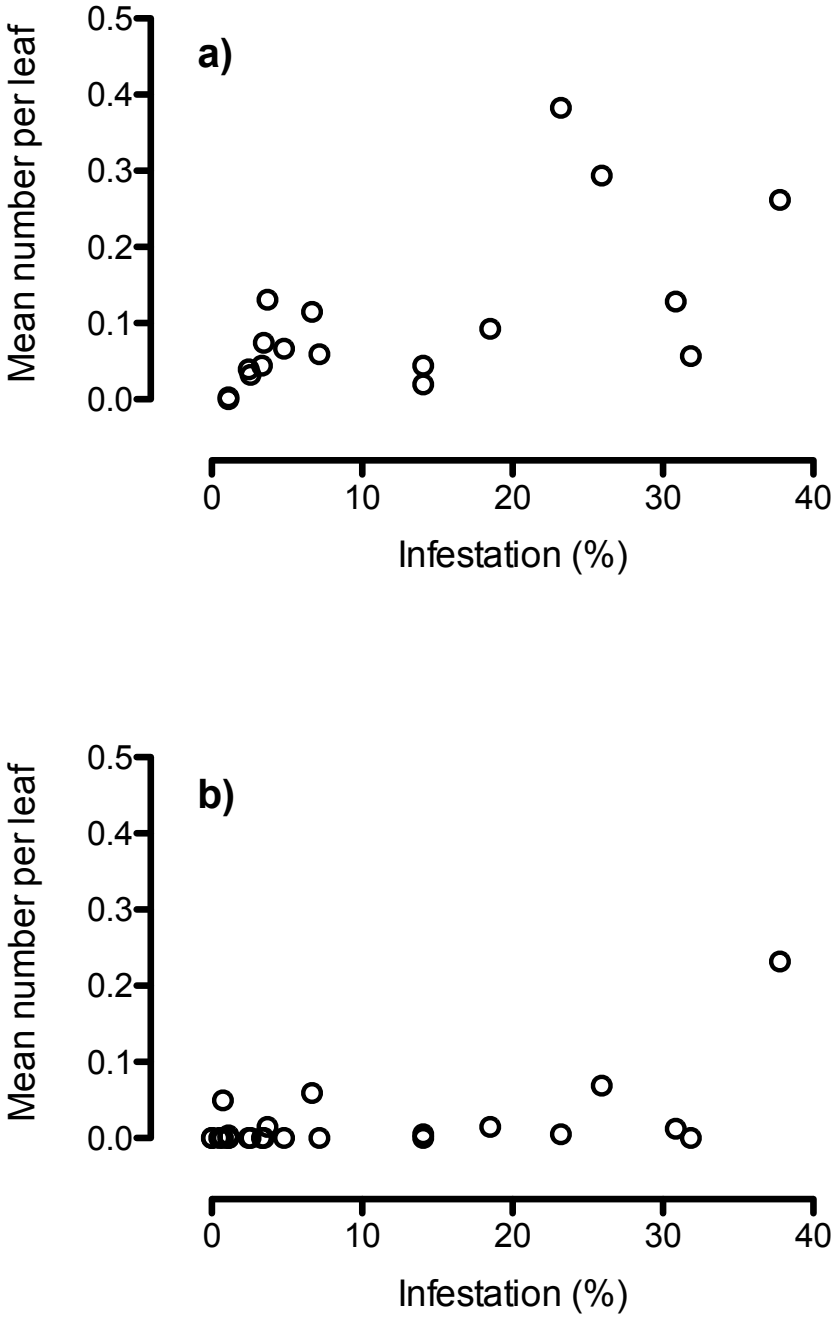


Figure 5 Spearman's rank correlation between aphid infestation (%) and **(a)** total density of predators (R=0.61 P=0.006,) and **(b)** density of mummies (R=0.44 P=0.03,). MB=materBi, RC=roller crimper, GM=green manure.

Beneficials were more abundant on the MB treatment, which was characterized by higher pest density. The predators belonged to the families: Coccinellidae, Anthocoridae, Syrphidae, Chrysopidae, Miridae and Cecidomidae. Overall, seven coccinellid species were found; the most abundant species was *Hippodamia variegata* Goeze (64.81%) followed by *Coccinella septempunctata* L. (9.26%) and *Propylea quatuordecimpunctata* (L.) (5.56%). *Harmonia axyridis* Pallas (1.85%) and *Ceratomegilla undecimnotata* Schneider (0.93%) were found only occasionally. Two phytophagous coccinellid species, *Psyllobora vigintiduopunctata* (L) (0.93%) and *Henosepilachna elaterii* (Rossi) (0.93%) were found, but not considered among predators. Scymninae accounted for the remaining 15.74% of the total coccinellids. *Macrolophus pygmaeus* (Rambur) was the most abundant Miridae found in all sampling dates.

Predator's density peaks were recorded on the third sampling date (65 DAT), showing a delay respect to the peak of aphid infestation (fig. 6 and 7). *Aphidoletes aphydymiza* (Rondani) was the dominant predator in 2015 showing a peak density of 0.7 larvae per leaf in MB plot (in one leaf a total of 23 *A. aphidimyza* larvae was found on a aphid colony). Other predators, like syrphids and coccinellids, were less abundant in 2015 in comparison with 2014 (fig 6 and 7).

Aphid parasitoids were recorded on aphid colonies with a higher mean number of mummies in 2014 than in 2015. Furthermore, only in 2014 the peak of mummy density was registered on the second sampling date (48 DAT), showing an early appearance of parasitoids in comparison with predators. On the other hand, in 2015 the highest density of mummies was concomitant with the peak of predators (48 DAT). Finally, the yield mean value (kg/plant) during 2014 was: 3.75 in MB, 1.38 in GM and 1.03 in RC. A similar trend was observed in 2015: 4.23 in MB, 2.70 in GM and 1.04 in RC.

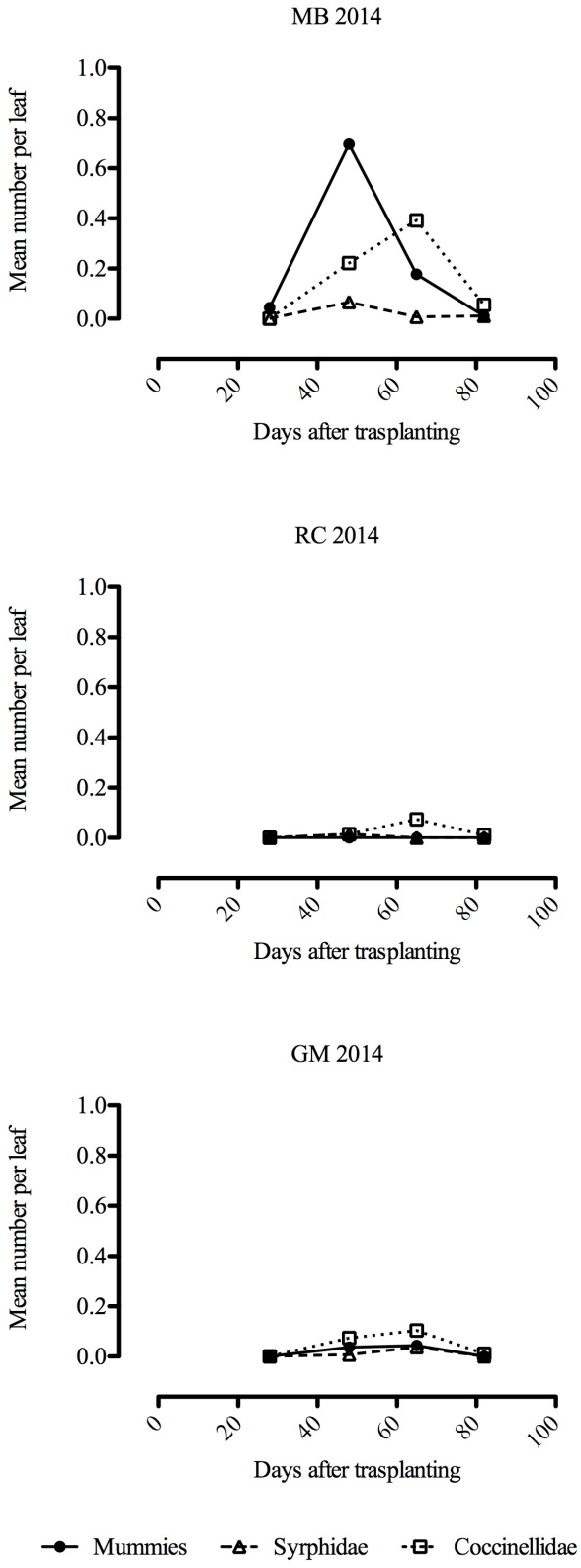


Figure 6 Individual per leaf of natural enemies in 2014. Only the aphidophagous instar of Syrphdae (larvae) and Coccinellidae (adults and larvae) were considered in this graph. MB=synthetic biodegradable film covering, RC=roller crimper, GM=green manure.

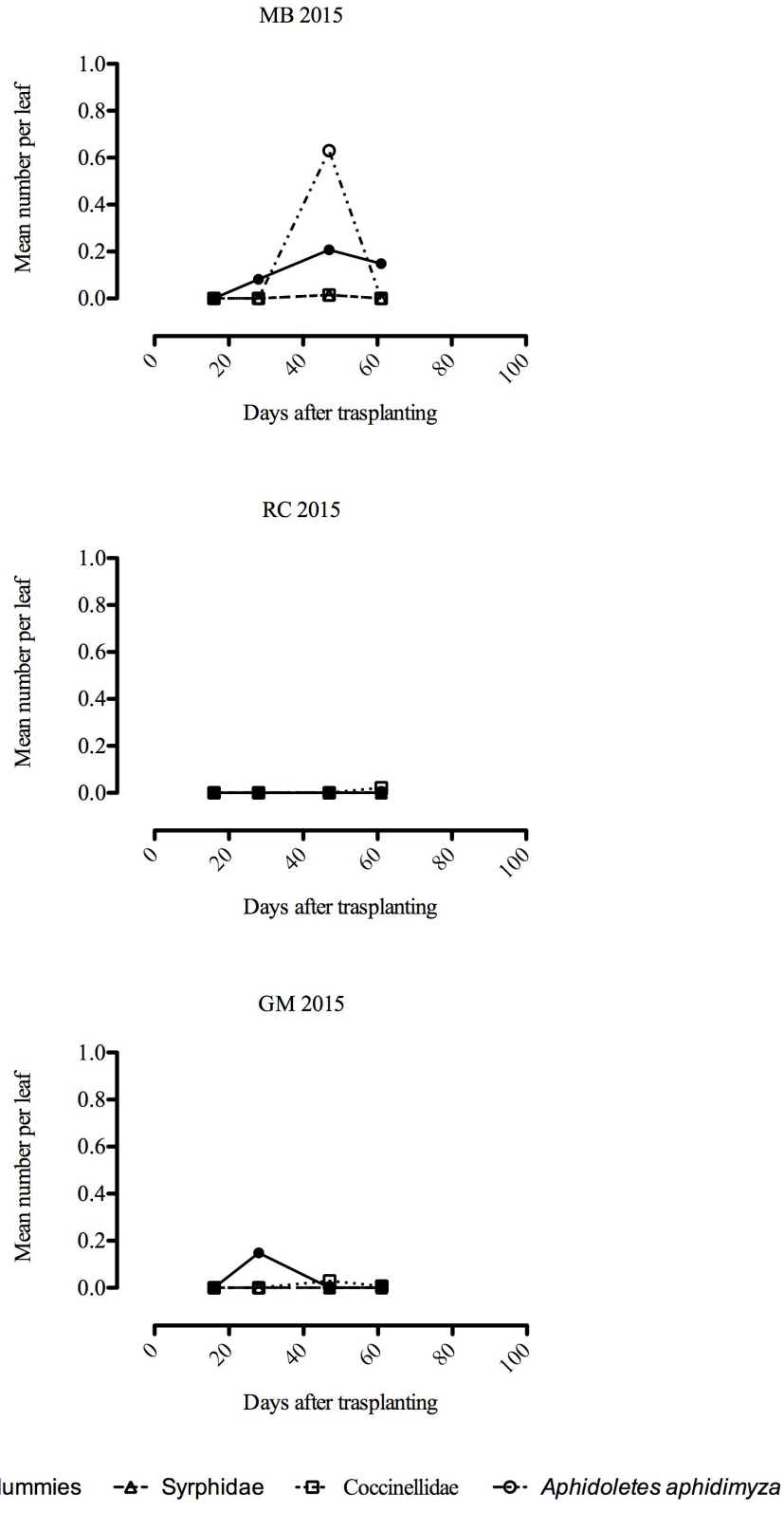


Figure 7 Individual per leaf of natural enemies in 2015. Only the aphidophagous instar of Syrphdae (larvae) and Coccinellidae (adults and larvae) were considered in this graph. MB=synthetic biodegradable film covering, RC=roller crimper, GM=green manure.

4.5. Discussion and conclusions

A.gossypii was the only relevant pest in our study. The lower aphid infestations were observed in the two ASC termination techniques (GM and RC) and it might be related to the lower vegetative growth. Our experiment demonstrates that the biofilm utilized in MB for weed control, strongly affected the vegetative development of plants. The most abundant vegetative growth and yield in MB plot could be explained considering the higher nitrogen nutritional status of the plants and the N content within plant tissue of this treatment, as evidenced by the indirect measure obtained from the chlorophyll content of leaves (SPAD index, data shown in fig.3). The lower yield mean value measured in the GM and RC treatments might be influenced by the poor adaptability of the *cultivar* "zuboda" to grow in low N availability conditions, as those generally occurring in soils after the cultivation of a non-lugume ASC (Thorup-Kristensen, 1993).

The higher leaf N concentration of MB plots, combined with the soil temperatures, could be the reason of the more abundant vegetative growth in this plot, resulting in higher aphid infestation in comparison with those of the ASC termination techniques (RC and GM). These results (fig.2) are in accordance with the literature (Truax and Gagnon, 1993; Teasdale and Abdul-Baki, 1995), indicating that the temperature lower in soil characterized by conservation techniques leads to slower growth and the lower vegetative vigor in GM and RC plots can be correlated to the lower aphid suitability of the plants belonging to these treatments.

Beneficial abundance was higher in the more infested MB plots, likely contributing to the control of aphid infestation. During the 2014 season, high densities of hymenopteran parasitoids were counted in MB in correspondence of peaks of aphid infestation, showing a good synchronization of the parasitoids with the aphids. In 2015, *A. aphidimyza* was the most abundant predator in MB plots; a strong density dependent numerical response of this

predator against its prey was observed. Adult emergence represents a very sensitive phase in the life cycle of *A. aphidimyza.* In particular meteorological condition, like dry weather, can strongly affect their possibilities to survive (Havelka and Zemek, 1988; Vacante and Benuzzi, 2007). These factors could explain the variability between years in the occurrence of this predator. Also predators like Coccinellidae, Anthocoridae, Syrphidae, Chrysopidae and Miridae could have concurred to control aphid infestations.

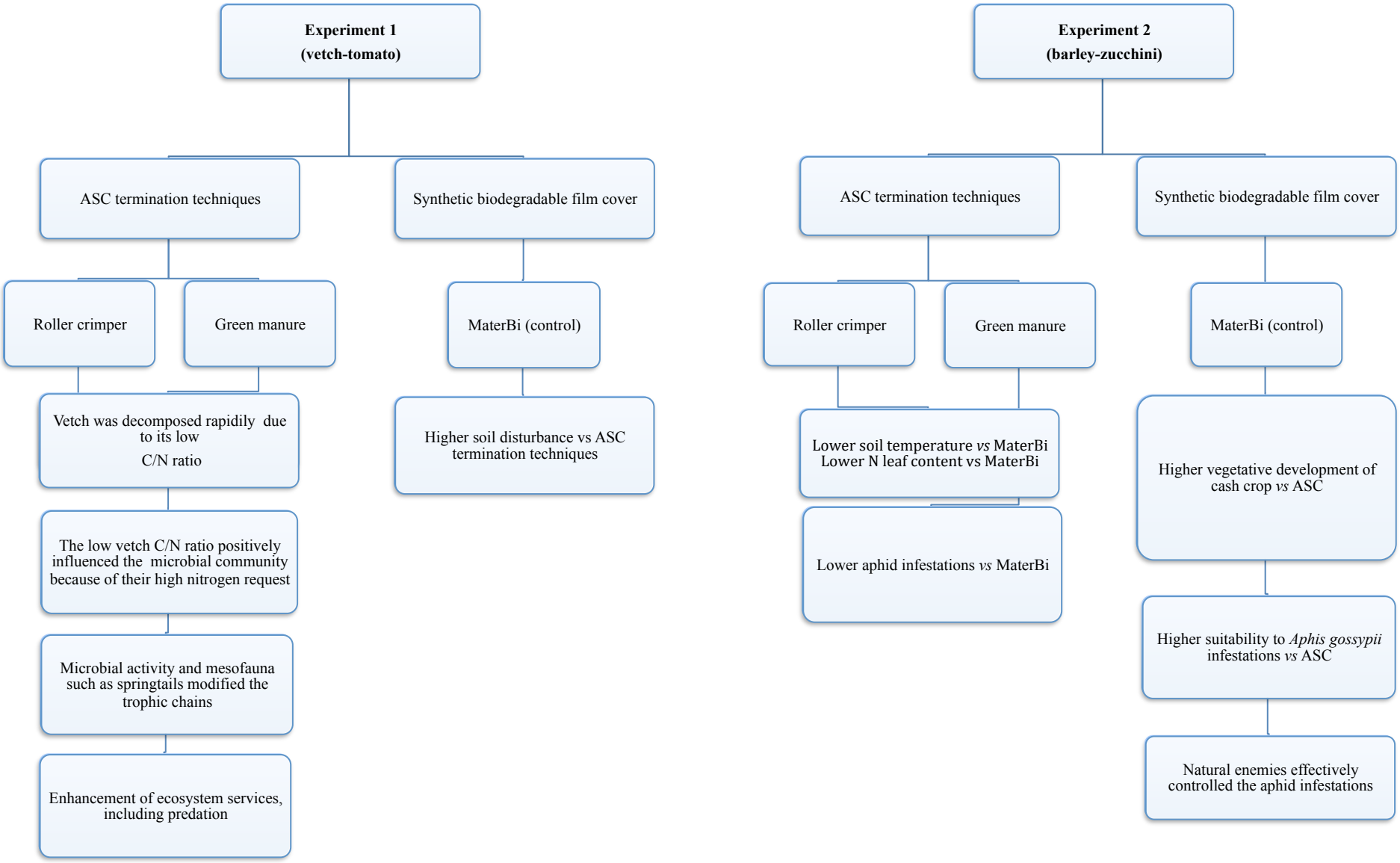
Our study was finalized to a practical evaluation of conservation biological control in an organic farm, which was not subjected to any sprays. From our experiment, a positive correlation between density of natural enemies and aphid infestation was shown; moreover no plant was seriously damaged by *A. gossypii*.

Overall, our results are consistent with other studies focused on the role of natural enemies against *A.gossypii* (Turchin and Kareiva, 1989; Burgio et al., 1994; Ferrari and Burgio, 1996; Al Hassan et al., 2012; Iguchi et al., 2012;).

In conclusion, MB treatment increased aphid infestation in comparison with both the ASC termination techniques (RC and GM). For this reason, the techniques tested in this experiment, and in particular RC system, may be suggested as a tool to mitigate aphid infestation.

Further study should be done in organic vegetable cropping systems in order to better understand the interactions between the agronomic variables and ASC termination techniques and their effects on pest and beneficial insect dynamics. Yield, weed control, microorganisms and other aspects related to ASC management and terminations were also considered during this study, but they will be discussed in subsequent articles. Aspects related to microbial community were reported in (Manici et al., 2016).

Flow chart reported at the end of this chapter summarizes the main results obtained by ORTOSUP project (chapter 3 and 4 of my thesis).



Flow chart 1 Summary of the main results obtained within ORTOSUP project (chapter 3 and 4 of my thesis).

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4.6. Acknowledgement

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5. Molecular detection of the olive orchards pest *Bactrocera oleae* (Diptera: Tephritidae) within gut contents of *Ocypus olens* (Coleoptera: Staphylinidae)

5.1. Abstract

Olive fruit fly *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) is one of the most important pests in olive orchards causing yield losses and affected oil quality. Despite the development of larvae occurred within drupe, during autumn the third instar escapes for pupate in the ground where will remains in hibernation until the next Spring. During this phase pupae are susceptible to predators. Samplings were carried out from October 2015 to March 2016 in five olive orchards between the province of Pisa and Lucca in Tuscany region. Molecular analyses of predation were carried out on 118 specimens of *O.olens* (Coleoptera: Staphylinidae) with the purpose to elucidate the role of this generalist predator towards *B.oleae*. PCRs (Polymerase Chain Reaction Amplification) were carried out on *O.olens* gut contents using specific primers already published (Rejili et al., 2016) for the detection of *B.oleae* DNA. On the total of specimens analyzed, 24.58% were positive to DNA of *B.oleae* confirming the importance of *O.olens* as a predator of the olive fruit fly.

Keywords: *Ocypus olens*, *Bactrocera oleae*, predation pressure, gut contents, PCR

This paper is in preparation for BioControl as:

Albertini A., Marchi S., Petacchi R., Burgio G., Ratti C., Magagnoli S. - Molecular detection of the olive orchards pest *Bactrocera oleae* (Diptera: Tephritidae) within gut contents of *Ocypus olens* (Coleoptera: Staphylinidae).

5.2. Introduction

One of the major agro-ecosystem in the Mediterranean Basin is olive orchard (*Olea europaea* L.*)*, of utterly socioeconomic and dietary values (European Commission, 2012). Recently, multifunctionality provided by this agro-ecosystem have been investigated (Fleskens et al., 2009), pest control service being emphasized (Simon et al., 2010; Rodriguez-Saona et al., 2012; Gkisakis et al., 2016). The most destructive pest of olive orchard is the olive fruit fly *B. oleae* (Rossi) (Diptera: Tephritidae), a monophagous frugivorous species that feeds exclusively from *Oleae* spp. (Tremblay, 2003). Its infestation can cause dramatic economical losses, affecting the entire olive chain, from farmers to industrial and consumers (Malheiro et al., 2015). Because of its almost worldwide distribution and a growing resistance to pesticides (Daane and Johnson, 2010), there is an increasing interest in developing new strategies to control this pest. Environmentally friendly efforts prompt in the direction of biological control methods targeting the pupal stage of *B. oleae*. This polivoltine fly lays eggs inside the drupe, where the metamorphosis is completed until the adult open an exit hole. However, for the late summer-early autumn generations, third instar larvae generally escape from the drupe and pupate within a few days in the ground, remaining in diapause until the following spring (Bateman, 1972; Cavalloro and Delrio, 1975). Here in the top soil, hibernating pupae are vulnerable to soil predators (Dimou et al., 2003), generally referred to staphylinids, carabids, ants, earwigs, spiders, opiliones and miriapoda (Lasinio and Zapparoli, 1993; Orsini et al., 2007; Dinis et al., 2015). Heterogeneity of the predatory guild is attributed to several factors, among which the localization of the orchard, the management regime and the surrounding vegetation (Morris et al., 1999)

Staphylinids (Coleoptera: Staphylinidae) are one of the largest family of Coleoptera with over 55.000 described species (Lott and Anderson, 2011). The family is worldwide

spread, but they are difficult to identify at the species level and their biology and general life history are not well known. They may play a relevant role in agro-ecosystems in the light of their niche differentiation, as they can act as predators, detritivores, herbivores and fungivores (Clough et al., 2007). In addition, some predators are known to disperse and colonize both the cropped field and the surrounding semi-natural habitat (SNH), thus providing ecological services at different scales (Dennis and Fry, 1992; Andersen, 1997). Some staphylinids species have been identified as potentially important natural enemies of pests within arable crops (Winder et al., 1994; Symondson, 2004), but knowledge of their ecology in rural environments are extremely scarce. Several evidences suggest that staphylinds may have impact on the mortality of *B. oleae* pupae (Dinis et al., 2015). In olive orchard agro-ecosystem, high abundance of these beetles has been frequently reported, particularly during the months when *B. oleae* pupae are more abundant in the top soil (Lasinio and Zapparoli, 1993; Tzokas et al., 2004; Gkisakis et al., 2016). However, to our knowledge, no studies were performed to test the effectiveness of staphylinids in *B. oleae* pupae consumption.

Generally speaking, several hurdles makes arable food web difficult to be unraveled: spatio-temporal fluctuations in both predators and prey densities, farming practices and management intensity and semi-natural habitat features (Rusch et al., 2013). Nowadays, DNA-based approaches have proved to be effective tools for such cryptic arthropods trophic webs, and prey's target sequences are detected from the semidigested remains in the predator's gut (Zaidi et al., 1999; Symondson, 2002; Harper et al., 2005). Polymerase chain reaction (PCR) analyses of gut content have been used for a number of diet assessment on soil predators of agricultural interest, mainly carabids (Monzó et al., 2011; Pianezzola et al., 2013; Staudacher et al., 2016). Since the likelihood of detecting the prey target sequence decreases with increasing digestion time, one of the main ecological issue

is addressing at the maximum time to which the prey consumption can be detected (i.e. the prey DNA is amplifiable from the predator's gut) from the end of feeding activity, in order not to misestimate predation.

In this regard, the present chapter investigates *B. oleae* pupae detection in staphylinids that were directly collected in olive orchard agro-ecosystem. This implied we could not know if the staphylinids had *a priori* eaten *B. oleae* pupae, and, if it was the case, if the predation occurred in a detectable molecular time. We focused on one species, *Ocypus olens* (O.F. Müller, 1764), a large (adult length approximately 17-33 mm) black staphylinid, easy to handle and identify. This species, indigenous to Europe, has an opportunistic diet (Nield, 1976) and can be a voracious predator with high prey consumption (Bonacci et al., 2006). Despite its role as predator, the potential of this species for pest control has not still tested exhaustively and only few records was found on this topic in the literature (Orth et al., 1975; Fisher et al., 1976).

We aimed at i) testing the methodology of PCR gut analysis in specimens collected from field; ii) detect possible seasonal variations and differences among fields in the predation rate.

5.3. Material and Methods

5.3.1.1. *Study sites*

Field work took place in Tuscany, one of the top region in Italy for quality olive oil production, and the first one for protected designations of oil and protected geographical indication oil production, both in term of total area and number of holdings (Adua, 2010). Sampling stations were in Monte Pisano area, North-West Tuscany, a small hilly region (15200 ha, highest peak of 917 m asl) devoted to olive oil cultivation run following old traditional practices. The climate is typically Mediterranean, with annual mean

temperature about 14.3 °C and annual average precipitation 1106.9 mm (Niccolai and Marchi, 2005). Monte Pisano landscape is an agricultural mosaic, interspersed in patches of ancient mesophilic woods and Mediterranean garigue semi-natural habitats (SNHs). As the type of SNH can affect species activity and predation rates (Bianchi et al., 2006; Picchi et al., 2016), we chose olive orchards that were all surrounded by woody SNHs ("woody areal type according to Holland et al., 2016). Five olive orchards located between the province of Lucca and Pisa were investigated (tab.1).

Table 1 Information about olive orchard investigated

Olive orchard	Province	Coordinates
Agnano	Pisa	43°44'25.8"N; 10°28'25.7"E
Montemagno	Pisa	43°43'11.9"N; 10°32'02.5"E
Rigoli	Pisa	43°47'08.4"N; 10°25'58.0"E
Ruota	Lucca	43°45'53.4"N; 10°35'15.2"E
Pieve di Compito	Lucca	43°47'02.4"N; 10°34'08.0"E

The orchards had an average area of 1 ha and laid between 200 and 300 m asl. Autochthon Frantoio and Leccino were the predominant cultivars, planted with a tree density between 250-300 trees per ha. In this Tuscany area, olive cultivation is based on integrated pest management (IPM) strategies and follows the Rural Development Programme for Tuscany Region (Reg. (UE) 1305/2013). *B. oleae* is recognized as the key pest and the orchards are part of the regional network for monitoring the pest activity and infestation level (Marchi et al., 2016). All orchards were non-irrigated, non-tilled and subjected to weed mowing. No herbicides were used and a spontaneous herbaceous layer was preserved through the year. Woody SNHs were mainly composed by i) pine trees (*Pinus pinaster* Aiton) with understorey vegetation dominated by either *Pteridium aquilinum* (L.) Kuhn or *Rubus* spp. or ii) mixed stands dominated by *Quercus* species (mainly *Q. ilex* L. and *Q. pubescens*

Wild.) with a poor understorey vegetation composed by *Erica arborea* L., *Arbutus unedo* L., and *Smilax aspera* L.

5.3.1.2. *Staphylinids collection*

Staphylinids were collected from October 2015 to March 2016. According to *B. oleae* life cycle, pupae are mostly present in the soil from late Summer to early Spring. Staphylinids were collected alive by means of modified semi-dry pitfall traps. The use of solutions in pitfall traps was avoided because some preservative used for soil predator collection, such as acid acetic or propyleneglicol, are known to inhibit PCR (Gurdebeke and Maelfait, 2002; King et al., 2008). Therefore, dry pitfall traps, i.e. without any preservative, have frequently been suggested. We run a preliminary test with plastic dry pitfall traps during October and November 2014 in the mentioned olive orchards. Staphylinids collection was extremely scarce. Thus we modified the trapping system as follows. Each modified dry pitfall trap consisted of 2 plastic vessels (each 85mm in diameter at the mouth and 120 mm deep) one within the other, so that a space of around 1.5 mm was created between the two bottoms. The bottom of the upper vessel had 4 small holes (3 mm in diameter) equally distant. We put 40 ml of acetic acid [gl^{-1}], so that the liquid did not exceed in the upper vessel, but the attractiveness could still be exerted through the holes. The system was dug into the soil to the outer edge of the upper vessel.

For each site, 15 modified dry pitfall traps were located along a transect, with a distance of 6-10 m from each other. To avoid field margin effect, the sampling points were positioned in the central field area.

Predators are usually active during night (Lövei and Ferrante, 2016) and thus, traps were activated in the afternoon and checked the morning after, so to guarantee a digestion time not exceeding 20 h. In laboratory, staphylinids were individually frozen at -80 °C for

subsequent molecular assay. *O.olens,* was selected for this study both for its large body size and because easy to identify.

Moreover, a preliminary feeding assay was carried out in laboratory on six specimens of *O.olens* starved for 48 hours and then fed with one pupae of *B.oleae*. Individuals were frozen at different periods (2, 6, between 8-15 and 15-20 hours) in order to acquire some information about their digestion time.

5.3.1.3. Primers, DNA extraction and amplification

DNA was extracted using the whole insect deprived of its head, legs and elytra. Extraction was performed with the CTAB method (Doyle, 1990) conveniently modified in order to improve the efficiency of DNA extraction.

Insects were individually placed in 1.5 ml tube with 700 µl of "Doyle & Doyle" grinding buffer (2.7 M CTAB, 1.2 M NaEDTA, 0.06 M NaCl, 3.8 M Tris-HCl), ground with a sterile pestle and incubated at 60°C for 30 minutes. To each sample were added 700 µl of chloroform-isoamyl alcohol (24:1). Tubes were centrifuged for 5 minutes at 13,200 g and the supernatant (approximately 500 µl) was incubated with 500 µl of cold isopropanol (-20°C) for at least 15 minutes at -80°C. Samples were centrifuged for 15 minutes at 13.200 g, the supernatant was removed and the pellet washed with 500 µl of cold 70% EtOH. After brief centrifugation (5 minutes at 13,200 g), pellet was resuspended, in 400 µl of TE buffer, 20 µl of NaOAc (4M) and 900 µl of cold 100% EtOH then incubated at -80°C for 10 minutes. The supernatant was discarded after centrifugation at 13,200g for 15 minutes and the pellet was washed with 500 µl of cold 70% EtOH as before. Finally, the pellet was dried under vacuum and dissolved in 100 µl of nuclease free water.

The primer pair SBo2-F/SBo1-R was used to specifically amplify a 214 bp long region of the *B. oleae* cox1 gene (Rejili et al., 2016). Sequences of primers were reported in the table 2.

Table 2 Sequence of primers SBo2-F/SBo1-R

Primer	Sequence
SBo1-R	5′ CTG GGT CGA AAA AGG AAG TAT 3′
SBo2-F	5′ TTA GCA GGT ATC TCC TCA ATC 3′

PCR reaction mix was prepared in a final volume of 25 µl with 5 µl of extracted DNA, 5 µl of 5X Colorless GoTaq® Reaction Buffers (Promega), 1.5 µl of $MgCl_2$ (25 mM), 0.5 µl of Mix dNTPs (10 mM), 1 µl of SBo2-F primer (10 µM), 1 µl of SBo1-R primer (10 µM), 0,2 µl of Go Taq DNA polymerase (5U/µl) and 1.25 µl of BSA (Bovine Serum Albumin).

PCR program was characterised by denaturation for 3 minutes at 94°C, followed by 30 cycles of amplification: 30 seconds at 94°C, 40 seconds at 60°C, and 1 minute at 72°C. A post-PCR final elongation for 10 minutes at 72°C closed the amplification process. DNA extracted from *B. oleae* pupae was included as positive control while DNA extracted from psyllids was used as negative control in order to exclude hidden contaminations.

All PCR products were separated on agarose gel (1.5%) and visualized under UV light after staining with ethidium bromide (fig.1).

Six positive samples were sequenced in order to confirm the primers specificity. Aliquots (2-3 µl) of positive PCR reaction were cloned using the p-GEM-T Easy Vector System (Promega). *Escherichia coli* cells (strain MC1022) were transformed by electroporation, plated on LB-plates containing ampicillin (100µg/ml), X-Gal (80µg/m) and IPTG (0.5mM) then incubated overnight at 37°C. Recombinant plasmids were isolated using the Wizard® Plus SV Minipreps DNA Purification System (Promega) following the manufacturer's instructions and sequenced by Eurofins Genomics (Germany).

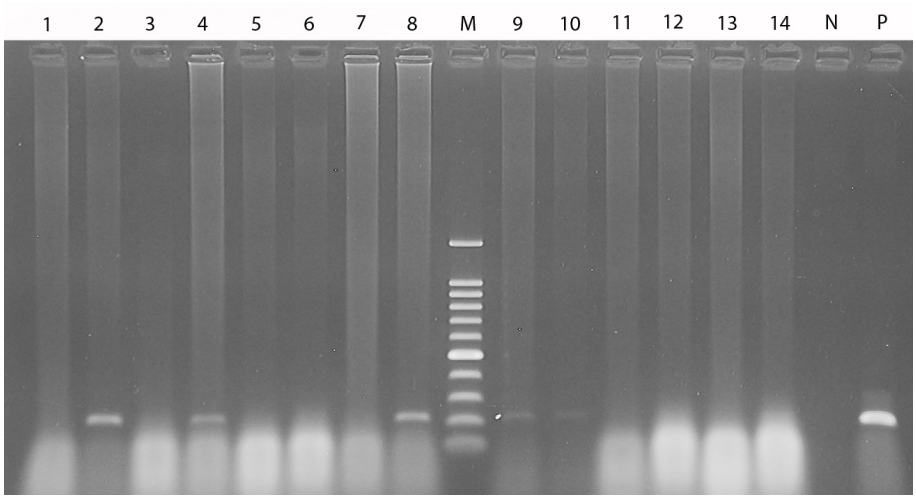

Figure 1 PCR products were separated on agarose gel (1.5%) and visualized under UV light. Specimens of *O.olens* number 2, 4, 6, 7 and 8 were positive to DNA of *B.oleae*. Letter "M" is 100 bp DNA ladder, while letter "N" and "P " are the negative and positive control respectively.

5.4. Results and Discussion

Ocypus olens was the most abundant species, accounting for 92.58% of the Staphylinidae collected (n=212) during olive orchard sampling. The high dominance of this species within staphylinid community, and the large body size, confirmed its suitability as a target species for *B.oleae* detection.

Preliminary PCRs were carried out on the six *O.olens* specimens screened in the feeding assay resulted positive to *B.oleae* for all the digestion time investigated (fig.2).

Gut content analyses were carried out on 118 field-collected specimens of *O.olens.* The total percentage of *O.olens* positive to DNA of *B.oleae* was 24.58%, confirming the role of this staphylinid species as a predator of the olive fruit fly. The positive predation rate in each olive orchard and in the different sampling periods is reported in fig.3 and fig.4 respectively. Tab. 3 shows the positive and negative *O.olens* on the screened specimens. The primers (Rejili et al, 2016) used in this study amplified a fragment of the mitochondrial gene cox1 (fig.5), characterized by high variable regions among different animal species. Thus, on the basis of the sequences divergence within cox1, is possible to confirm its suitability to identify *B.oleae* target among potential preys within gut content.

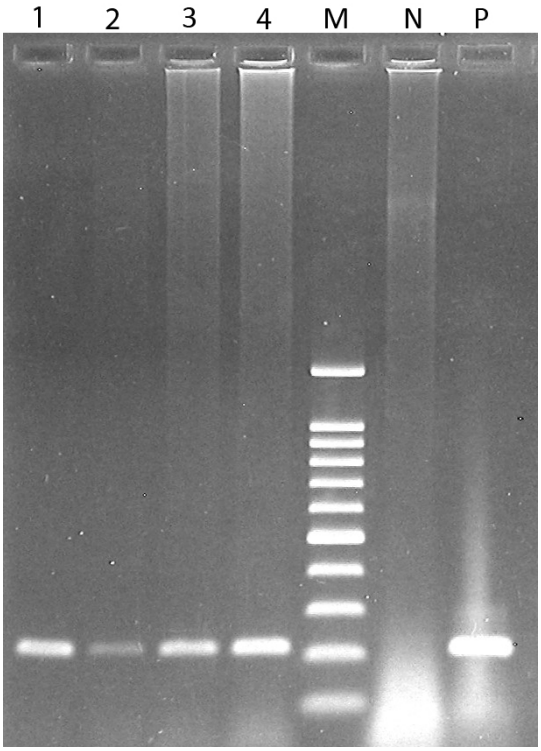

Figure 2 Visualization of DNA extracted from specimens frozen at different digestion time (**1**= after 2 hours, **2**= after 6 hours, **3**=between 8-15 hours, **4**= between 15-20 hours) on agarose gel. Letter M is 100 bp DNA ladder, while N and P are negative and positive control respectively.

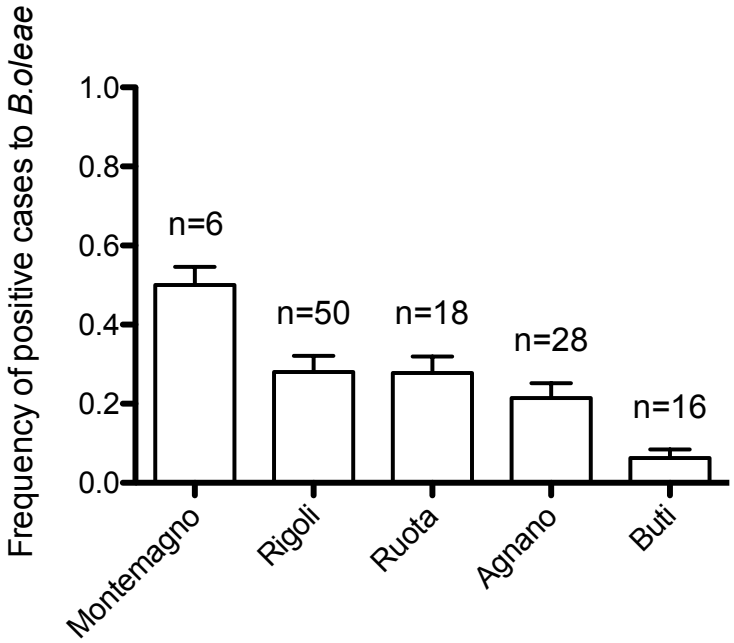

Figure 3 Frequencies of *O.olens* positive to DNA of *B.oleae* per each olive orchards. Bars represent SE of binomial distribution.

Table 3 Number of *O.olens* positive and negative to *B.oleae* per each sampling collection period.

Sampling period	Positive	Negative	*O.olens* screened
October	0	12	**12**
November	23	47	**70**
December-March	6	30	**32**
***O.olens* screened**	**29**	**89**	**118**

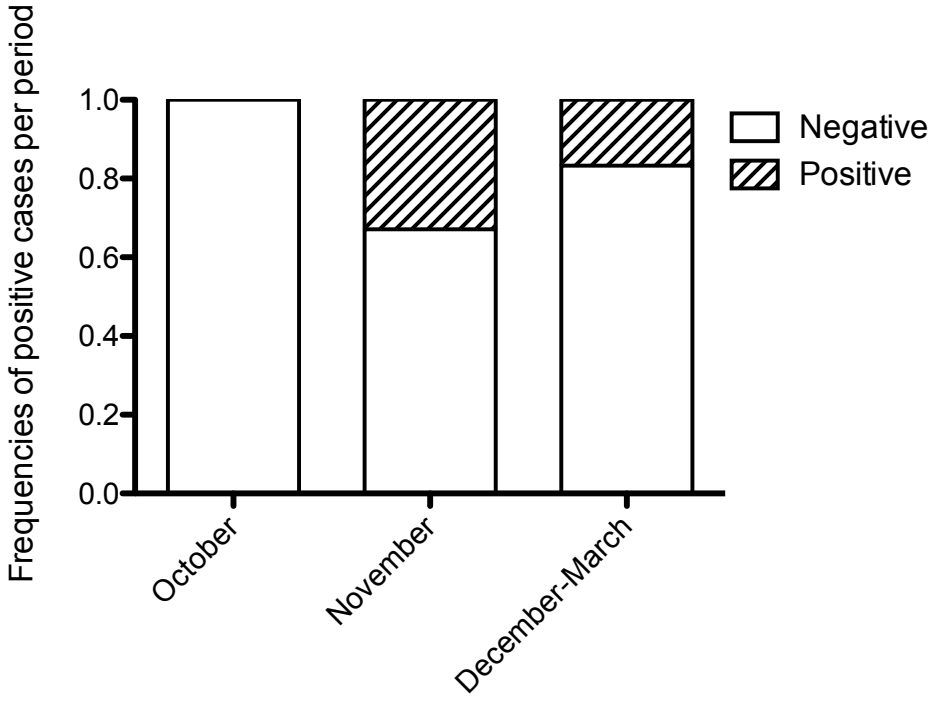

Figure 4 Frequencies of *O.olens* positive to DNA of *B.oleae* per each seasonal period.

Predation is one of the most important ecosystem services provided by arthropods within agro-ecosystems. However, despite predation pressure is still difficult to evaluate in field conditions, molecular approaches may supply information about the predator diet, providing a direct estimation of the predation pressure. In this study, the role of *O.olens* as

predators of *B.oleae* was confirmed by molecular analysis, highlighting the importance of this generalist predator in pest control. Moreover, our results are also corroborated by *B.oleae* pupae simulation model. Indeed, the presences of *O.olens* positive to olive fruit fly are overlapping with the output of the simulation model (data not shown). However, many variables may have influenced our finding. First of all the digestion of DNA prey by gut enzymes of predator that may have negatively decreased the *B.oleae* detection underestimating the predation rate. Second, the sensitivity of the molecular technique can affect the prey detection, because more sensitive molecular analysis should improve the detectability of DNA prey, increasing the estimate of predation rate. In this regard, during our study, we decided to carry out preliminary test to analyze few samples by nested PCR. The preliminary results confirmed my hypothesis, demonstrating a better sensitivity of this technique in comparison with the "conventional" PCR. However, one of the most common problems occurred using nested PCR is the contamination of samples caused by using during PCR reaction DNA already amplified. A crucial point is to assess if the higher number of positive samples by nested PCR is due by false positive or not, and a specific evaluation should be addressed on this topic. Also, further study will be addressed to deepen the digestion time of prey in the gut content of predators, in order to quantify the DNA decay rate. Moreover, a next step to complete this investigation could be the quantification of the DNA of *B.oleae* in the gut content of *O.olens* carrying out quantitative real time PCRs, in order to provide a quantification of the predation pressure of this species in olive orchard.

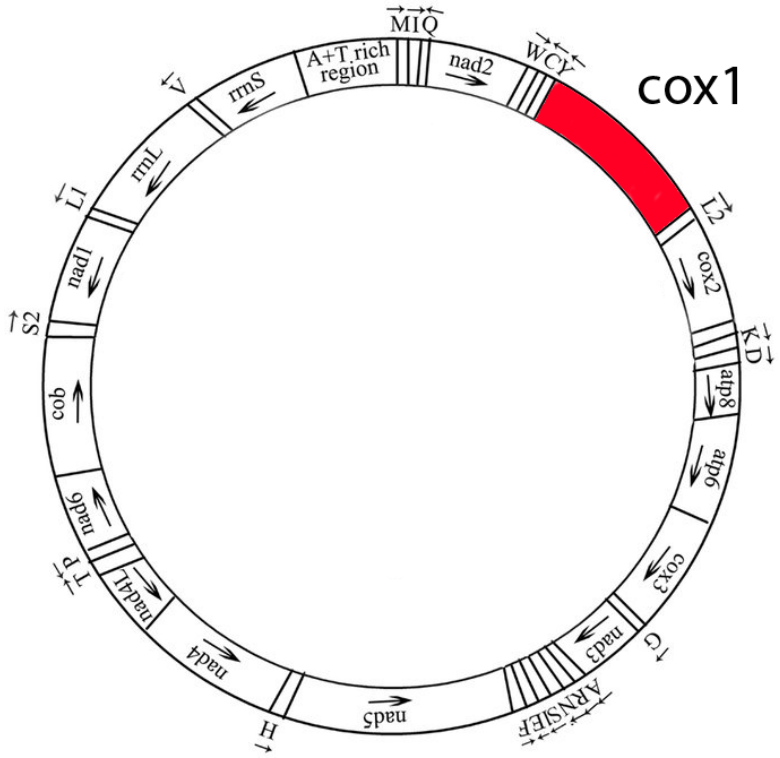

Figure 5 Mitochondrial DNA of insects. The primers used in this study for *B.oleae* amplified a specific fragment of gene cox1 (in red).

5.5. Acknowledgement

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5.6. References

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General discussion

The main purpose of my PhD was to improve the knowledge about functional biodiversity dynamics in an organic vegetable rotation characterized by different agroecological variables (chapter 3 and 4), and in olive orchard, to test the potential of Staphylinidae on *Bactrocera oleae* predation by molecular approach (chapter 5). Predation was selected as the target ecosystem service, for its important role in the trophic web of the studied system. The diverse community of predators were investigated during two consecutive years. A preliminary experiment was carried out in maize field to validate the potential of dummy caterpillar method to measure the predation pressure by soil predators (chapter 2). The major findings carried out from my work are summarised per each chapter:

Chapter 2

Title: Assessing predation pressure by artificial caterpillars: first validation for Italian agro-ecosystems

For the first time in Italian agro-ecosystems predation pressure was studied by using artificial caterpillars. During two consecutive years different maize fields, located near Bologna, were investigated with the purpose to evaluate the artificial caterpillar method. Our results are in agreement with the literature (see chapter 2), confirming this method as an inexpensive and easily realizable approach to study predation pressure. In conclusion, artificial caterpillars should represent a standardize approach to compare different soil predation rates among different conditions, including agricultural inputs and agroecological variables.

Chapter 3

Title: Influence of soil management techniques on predation pressure evaluated by artificial caterpillars.

Agro-ecological service crops (ASC) have an important role in sustainable agriculture, providing many benefits, but their impact on pest and natural enemy dynamics is still poor studied. Our experiments were carried out within a funded project (ORTOSUP) during two consecutive years in an organic vegetable systems located in Monsampolo del Tronto (Marche region, Italy). Two experimental areas characterized by different ASC (barley and vetch) and cash crop (zucchini and barley) were investigated. Moreover, two ASC termination techniques (roller crimper and green manure) were compared with a synthetic biodegradable film covering, the standard cultivation of vegetable in many Italian areas. Artificial caterpillars were used to assess predation pressure between the different experimental areas. The main results per each experiment are listed below:

Experiment 1 (Vetch-Tomato)

- Generalist predators, including carabids increased their activity density and predation pressure (measured by pitfall traps and artificial caterpillars method, respectively) in ASC termination techniques vs conventional cultivation.
- Among the termination techniques, roller crimper showed an higher soil predation in comparison with green manure.
- Microorganisms rapidly decomposed vetch (characterized by low C/N ratio) creating opportune habitat for soil organisms (i.e. springtails), favouring the abundance of preys and determining a positive cascade effect on generalist predators (carabids).

Experiment 2 (Barley-Zucchini)

- No differences in predation pressure were detected between agroecological techniques vs conventional, and between roller crimper termination and green manure.

- Graminaceous ASC created a thick layer of mulch that decomposed slowly in comparison with vetch; this could be the reasons of a general low abundance of functional groups and a lower predation rate measured by artificial caterpillars in this experiment.

Chapter 4

Title: Influence of agro-ecological service crop termination and synthetic biodegradable film covering on *Aphis gossypii* Glover (Rhynchota: Aphididae) infestation and natural enemy dynamics

In the same experimental areas, explained before (see chapter 3), we also studied the canopy insects occurred during two consecutive years, by sampling pest and beneficial dynamics.

However, despite visual samplings was employed in both systems, no significant infestation was recorded in "Vetch-Tomato" experiment. Below, our main findings are reported:

Barley-Zucchini system

- Plants in MB treatment were more susceptible to *Aphis gossypii* infestations in comparison with those characterized by cover crop termination.
- Graminaceous ASC steal nitrogen from the cash crop and for this reason the vegetative growth of plants cropped by agroecological techniques were lower in comparison with those cultivated by synthetic biodegradable film covering (MB).

  Also the higher soil temperature due by MB film likely concurred in increasing the vegetation growth of plants cultivated by MB techniques.
- Natural enemies were effective in controlling aphids in all the systems, including MB.

- My results demonstrated that ASC termination can strongly affect the soil-crop system, leading to a different suitability of the plant to aphid infestation. ASC termination could be considered a techniques to mitigate aphid infestation in the considered experimental condition

On the bases of results discussed in chapter 3 and 4 of my thesis, it's possible to conclude that use of ASC and ASC termination techniques are approaches very important for the sustainable agricultures, but their use is to consider with a case-by-case approach in order to maximize the benefits on pest-beneficial dynamics. Moreover, the new roller crimper technology represents an efficient technique to kill ASC and increase the soil ecosystem services, besides a positive role in weed control and reducing non-renewable inputs.

Chapter 5

Title: Molecular detection of the olive orchards pest *Bactrocera oleae* (Diptera: Tephritidae) within gut contents of *Ocypus olens* (Coleoptera: Staphylinidae)

The role of the generalist predator *O.olens* on *B.oleae* predation was investigated by using molecular approach. In this study, *O.olens* was the predominant species occurred in the samplings carried out in olive orchard system between October and March. Specimens were collected in different olive orchards with dry pitfall traps and frozen at -80° in order to avoid DNA degradation. PCRs were conducted on 118 specimens of *O.olens* showing high percentages of positive samples to *B.oleae.* This is the first demonstration of the role of *O.olens* in *B.oleae* predation. Results of this chapter stress the importance of the soil management of olive orchard for the Staphylinidae conservation, in order to increase the ecosystem services provided by this taxon.

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My research group at work. **N°1** Group shot (from the left to the right): Antonio Masetti, Giovanni Burgio, Serena Magagnoli (myself), Alberto Lanzoni and two master students. **N°2** Sweep net performed by Francesco Lami. **N°3** Giovanni Burgio & Gabriele Campanelli (behind with blue shirt) during a meeting for the ORTOSUP project. **N°4** Laura Depalo & Antonio Masetti in field to place pitfall traps. **N°5** Giovanni Burgio (rh) & Antonio Masetti (lh) while taking a break after work. N°6 Giovanni Burgio during "frappage". **N°7** Antonio Masetti & Laura Depalo in an artichoke field.