Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado em Engenharia Agronómica

Dissertação

The diversity of parasitoids in the Alentejo olive grove ecosystem and its potential contribution to the limitation of olive tree pests

Marina Favaro Quadrado

Orientador(es) | Fernando Manuel Rei

Tânia Mesquita Nobre

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A dissertação foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor da Escola de Ciências e Tecnologia:

Presidente	|	Vasco Fitas da Cruz (Universidade de Évora)
Vogais	|	Fernando Manuel Rei (Universidade de Évora) (Orientador) Maria do Rosário Félix (Universidade de Évora) (Arguente)

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Esta dissertação não é só minha, à todos vocês dedico este trabalho.

A diversidade de parasitoides no ecossistema do olival alentejano e o seu potencial contributo na limitação de pragas do olival

Resumo

A oliveira é uma cultura afectada por diversos insectos. Os organismos auxiliares naturais têm acção limitante sobre as espécies fitófagas e nesse grupo, a ordem Hymenoptera destaca-se por estar associada a muitos insetos parasitóides de fitófagos. Para melhor se conhecer a sua abundância e diversidade no olival, procedeu-se uma amostragem no Alentejo, em olivais não intervencionados quimicamente. Em cada local, insectos associados a oliveiras e plantas espontâneas foram amostrados. Diferenças significativas na sua abundância e diversidade, em função de várias variáveis ecológicas foram avaliadas (Kruskal-Wallis). Os resultados indicam uma maior abundância e diversidade de parasitoides na vegetação espontânea do solo, e as características da paisagem e a interação da precipitação e temperaturas parecem afetar a comunidade de parasitoides. Os parasitoides foram identificados morfologicamente e utilizando uma abordagem de 'DNA barcode', sendo composta principalmente por espécies amplamente generalistas, destacando-se algumas espécies por estarem referenciadas como parasitoides de pragas do olival.

Palavras-chave: Olival, Hymenoptera, parasitóides, DNA barcode, IPM.

The diversity of parasitoids in the Alentejo olive grove ecosystem and its potential contribution to the limitation of olive tree pests

Abstract

The olive crop is affected by several insects. The indigenous arthropod fauna have a limiting action on phytophagous species and in this group, the order Hymenoptera stands out for being associated with many phytophagous parasitoid insects. To better understand its abundance and diversity in the olive grove, a sampling was carried out in olive groves with no chemical load. Insects associated with olive trees and cover crops were sampled throughout Alentejo. Significant differences in parasitoids abundance and diversity, due to several ecological variables, was accessed (Kruskal-Wallis). Parasitoids abundance and diversity were higher in ground cover vegetation and the characteristics of the landscape and the interaction of rainfall and temperatures seem to affect parasitoid community. The parasitoids were identified morphologically and using a 'DNA barcode' approach and was composed mainly by broadly-generalist species, with some species previously referred as associated to the main olive pests.

Keywords: Olive grove, Hymenoptera, parasitoids, DNA barcoding, IPM.

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1. Introduction

The olive tree, *Olea europaea* L., is mainly distributed in all regions of the world with Mediterranean climate. In these regions, olive growing is an activity with great economic and social importance. Portugal is an important olive-producing country in the European Union occupying the fourth position after Spain, Italy and Greece, with 740.151 tons of olive production per year (FAOSTAT, 2018).

World olive growing is estimated of around 1.000 million olive trees, occupying an area of 10.2 million hectares and more than 90% of the total area is in the Mediterranean basin. Spain (with 55% of production) is the world's largest olive oil producer country and together with Italy and Greece accounts for about 96% of EU olive oil production (IOC, 2017). Recently, and driven also by consumer's demands, the market is changing to accommodate not only quantity but also quality production. The demand for high quality olive oils have led to an increase of typical marks, awarded to high-quality olive oils produced from local varieties grown in well-defined geographical regions. Also, the demand for biological or sustainable production practices has increased in the last years, and the trend is expected to stay. These changes require new approaches in olive groves management practices, including in pest management.

There are several pests which can attack the olive grove, standing out as the most frequent ones are the olive fruit fly (*Bactrocera oleae* Rossi), the olive moth (*Prays oleae* Bernard), the black scale (*Saissetia oleae* Olivier), the olive psyllid (*Euphyllura olivine* Costa), the olive bark beetle (*Phloeotribus scarabaeoides* Bernard) and the olive thrips (*Liothrips oleae* Costa) (Teixeira *et al*., 2000). In the Mediterranean basin area, the olive fruit fly and the olive moth are considered key pests, for the losses that they may cause (Gonçalves & Andrade, 2012a; Nobre *et al*., 2018).

Among the methods used to manage these pests, chemical control measures are the most widely applied. However, because of the detrimental effects of these chemicals on the environment and beneficial insects, in recent years, high socioeconomic pressures are forcing olive growers to develop alternative control strategies in an effort to mitigate the

undesirable side effects of pesticides on trophic chains and biological balances (Nave *et al*., 2017).

In the Mediterranean region, the traditional olive grove agroecosystem is characterized by a good stability, where there is a large complex of beneficial insects that may help to reduce pest population numbers (Bento *et al*., 1998). The natural control exerted by parasitoids seems especially promising, since these beneficial species constitute a large and relatively diverse group, whose efficiency can reach high levels in some regions (Nave *et al*., 2017). Studies conducted in Portugal have emphasized the frequency with which parasitoids are observed in olive groves, especially those belonging to the Aphelinidae, Braconidae, Encyrtidae, Eulophidae, Ichneumonidae, Pteromalidae and Trichogrammatidae families (Hymenoptera) (e.g. Serrano, 2016; Rei, 2006 Teixeira *et al*., 2000).

The functional fauna biodiversity of a given orchard depends on several factors, ranging from climactic, landscape and local structures that characterize the food-webs within the agrosystem. Thus, knowledge on the diversity of entomofauna in olive groves will enable a better understanding of the ecosystem (Torres & Bueno, 2000). Conservation of auxiliary entomofauna is one of the approaches of biological control in agricultural crops, namely natural limitation, and olive groves are no exception within this context (Amaro, 2003).

To achieve the goal of promoting natural pest limitation as a way to reduce pesticide use, it is necessary to know the indigenous fauna structure of the olive grove, as well as its spatial and temporal dynamics, to better understand, manage and protect the presence of entomophagous auxiliaries. In fact, identification and discrimination of the natural enemies and pest biotypes significantly increase the likelihood of success of natural limitation (Rei, 2006).

Therefore, this study was conducted to identify the pool of parasitoid species and to raise questions on their relative importance in the natural control of the olive main pests, and furthermore investigate the effects of ecological variables on their populations' abundance, in the Alentejo region of Portugal. We particularly focused on the olive fruit fly -*Bactrocera oleae*- as the main key pest affecting the region (Nobre *et al*., 2019). The potentially specific parasitoid community is likely mainly active in spring and autumn, preferentially

parasitizing the last two larval stages and pupae (Rei, 2006). Spontaneous vegetation in the grove and surroundings can enhance both parasitoid longevity and fecundity due to the availability of nectar and pollen (Furtado *et al*., 2016). The assessment was performed in the autumm period, the period that corresponds to the transition between a more fruit related populations of olive fruit fly (where they are more subjected to the action hymenoptera parasitoids) and the soil associated period (larval and pupal stages in the soil, which are more subjected to predators).

2. Olive crop

2.1 The origin of the crop

The olive tree, (*Olea europaea* L.), is the only specie of the *Oleaceae* family with edible fruit, and one of the oldest cultivated plants, whose origin dates from 4000-3000 years a. C. in the Palestinian region (Bacelar *et al*., 2009). Cultivation expanded westward across the Mediterranean basin through several nations (Greeks, Phoenicians, and Romans) (Gouveia, 2002), and later, as consequence of the maritime expeditions to the Americas, the Portuguese also had an important role in the geographical dispersion of this crop (Galado, 2007).

Nowadays, the olive crop is also present in countries located in other continents such as Australia, Chile, United States of America, Brazil, Canada, Japan (Reis, 2014), China and Argentina (IOC, 2017), although it is considered that around 98% of the olive oil world heritage is located in the Mediterranean area (Civantos, 2008).

2.2 The olive tree

The olive tree, *Olea europaea* L., belongs to the order Oleales, which consists of a single botanical family (*Oleaceae*), but it includes several species distributed throughout the tropical and temperate zones (Bacelar *et al*., 2009). The genus Olea comprises 35 different

species, including *Olea europaea* L., which produces edible fruits (Barranco *et al*., 2004) and is one of the most important according to an economic perspective (Bacelar *et al*., 2009).

The species *Olea europaea* L. is subdivided into the subspecies *Olea euromediterranea oleaster* or *Olea oleaster*; *Olea euromediterranea sativa* or *Olea sativa*; *Olea europaea* subspecie *laperrini* and *Olea europaea* subspecie *cuspidata*. *Olea euromediterranea sativa* or *Olea sativa,* is the olive tree commonly cultivated, consisting of a large number of improved cultivars, multiplied by cutting or grafting (Rodrigues, 2003).

In terms of morphology the olive tree is a polymorphic tree, with a thick and tortuous trunk (Barradas, 1998), and a root system that generally extends from 15 or 20 cm to 80 cm deep (Garcia, 2000). Normally the size of the cultivated olive tree is medium, ranging from 4 to 8 meters height. Its canopy is rounded and tends to thicken due to the vertical ramifications that grow inside. However, the shape that each tree acquires is influenced particularly by pruning and both agronomic and environmental conditions to which it is subjected throughout its growth (Lobo, 2009).

It is a species of slow growth and persistent foliage, lasting between 1 to 3 years. The leaves are simple, complete and with a short petiole. The arrangement of the leaves in each node is in opposite position (Barranco *et al*., 2004). Its morphological characteristics allow it to minimize light interception and heat exchanges, promoting effective control of transpiration (Lobo, 2009). This anatomical feature of the leaf is a result of the adaptation of this species to arid environments, in the sense of protecting it against excessive water loss (Bacelar e*t al*., 2009).

Depending on the region in which they are cultivated, the olive trees bloom between the end of April and the beginning of June. Panicle-shaped inflorescences develop in the leaf axils of vegetative growth nodes of the year prior to flowering. In each inflorescence there are 10 to 40 flowers on average (Bacelar *et al*., 2009).

The inflorescence presents two types of flowers: the first is hermaphrodite or bisexual, composed of well-developed stamens and pistil; the second, known as staminiferous or male, has a rudimentary or absent ovary and cannot originate the fruit (Suárez *et al*., 2012).

The flowers are small, actinomorphic, with regular symmetry and are composed of two sepals, four petals, two stamens and one pistil. The olive tree is essentially alogamic (Bacelar *et al*., 2009).

The olive fruit is an ovoid drupe consisting of three main structures: endocarp (olive pit), mesocarp (pulp) and exocarp (epidermis). The set of these tissues is called pericarp (Barranco *et al*., 2004).

During the maturation process, lipids accumulate in the cells of the mesocarp and simultaneously vitamins, hydrocarbons, sterols, pigments, polyphenols, alcohols, waxes, ketones and aldehydes are formed which, in association with the lipids, will thicken the fat droplets (olive oil) (Lidon *et al*., 2007).

On average, the olive fruit is composed of 50% water, 1.6% protein, 22% olive oil, 19.1% carbohydrate, 5.8% cellulose and 1.5% mineral salts (Monteiro, 1999). In addition, the phenolic composition of the olive is complex, and varies depending on the variety, maturation stage, season, geographical region, and cultivation practices (Ghanbari *et al*., 2012). Oleuropein is the most abundant phenolic compound in the olive fruit, representing the major constituent of unripe, green olives (Andrews *et al*., 2003). This abundant secondary metabolite is an olive-plant-produced defensive compound (a bitter and otherwise toxic phenolic glycoside), that is at a higher concentration on unripe olives and decreases throughout the fruit maturation process (Nobre, 2019). The chemical composition of the olive pulp is thus an important aspect on oviposition fruit selection by the olive fruit fly as its larvae are strictly monophagous. In contrast to other frugivorous Tephritidae Diptera, which feed upon hydrolyzed compounds of decaying and ripe fruit, *B. oleae* has the unique ability to utilize olive proteins and other nutrients of the olive flesh, as well as cope with high levels of phenolic compounds (namely oleuropein), which can reach up to 14% of the dry fruit weight, particularly in the unripe (green) olives (Ghanbari *et al*., 2012).

The optimum temperatures for the vegetative development of the olive tree are between 10 ºC and 30 ºC. Above these temperatures, and in particularly above 35 ºC, the tree closes the stomata to regulate its temperature, which can lead to a stop in its development. For the olive tree, cold is considered a factor that promotes floral induction; it

is necessary low temperatures for vernalization to occur. This tree needs about 400-700 hours of cold for floral differentiation (Barradas, 1998).

Regarding the water needs of the crop, it requires 300 to 400 mm per year of rainfall for a good production. The olive tree has a great ease to adapt to any type of soil. However, like most crops, it prefers medium-textured soils. This type of soils are the ones that allows it to access an adequate aeration for its growth and root development; make use of a medium to high water retention capacity; and presents a permeability that prevents root asphyxia (Cordeiro, 2014).

2.3 Olive growing in the World and in Portugal

Olive grove is present in areas of the world with Mediterranean climate characteristics, in which the summer is hot and dry and the winter is temperate (Reis, 2014), therefore it is confined to the zones that lies between latitudes 30° and 45° in the northern and southern hemispheres (Casa do Azeite, 2018).

According to FAOSTAT (2017), the quantity of olives produced in the world was 20.872.788 tons, occupying a worldwide area of 10.804.517 hectares. The main olive-growing continent is Europe with 61.3% of the world's production, followed by the African continent with 18%, Asia with 17%, the American continent with 3% and finally Oceania with only 0.6% (Figure 1).

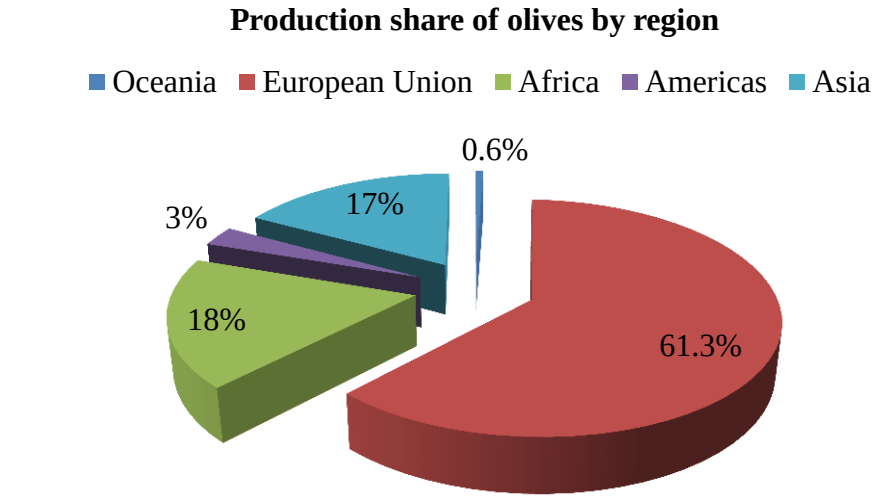

Figure 1: Production share of olives by region - 2016/2017 Campaign (Adapted from FAOSTAT, 2017).

Spain, Greece and Italy are the largest producers in the world (Figure 2), producing in the year of 2017, 6.549.499; 2.720.488 and 2.576.891 tons of olives respectively. Portugal ranks at eight amongst the world's largest producers; with a production of 876.215 tons of olives, and an average yield of 2.4456 tons/hectares (FAOSTAT, 2017).

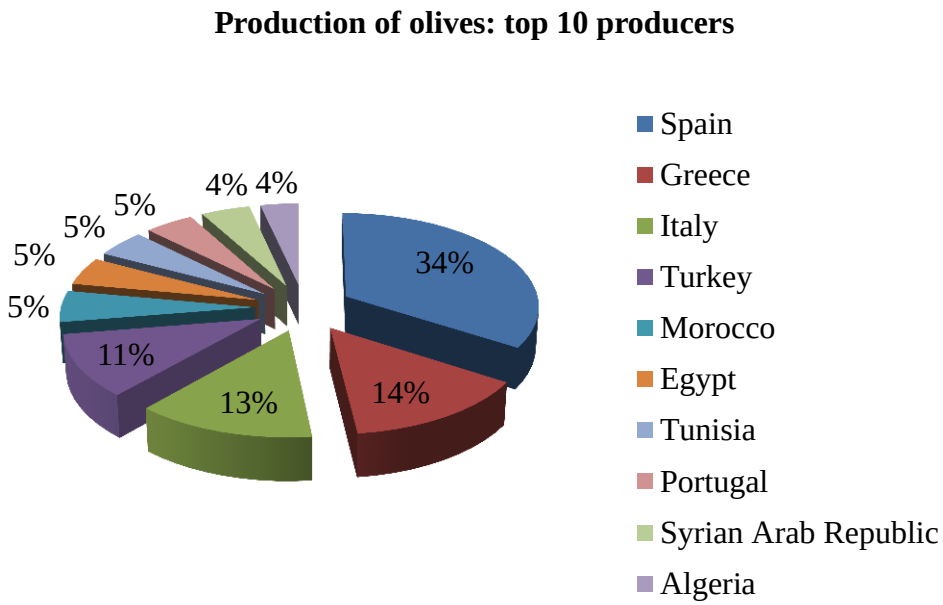

Figure 2: Main olive world producers - 2016/2017 Campaign (Adapted from FAOSTAT, 2017).

In Portugal the agricultural area extends by 336.000 hectares, being 97% destined to produce olive oil olives. Of the 351.000 planted hectares, only 8.800 hectares are for table olives (Casa do azeite, 2015). The largest olive grove in Portugal is in the Alentejo region (Table 1), with a national surface area of 52%, followed by the North region, with 23% ,and the interior region with 23% of the total area (INE, 2017). The olive grove is also present with greater importance in the regions of Beira Interior, Ribatejo and Trás-os-Montes (Cordeiro, 2014).

Table 1: Area and Production of olive groves in NUTS II (INE, 2017).

Region	Surface (ha)	Production (t)
Continent	356 183	493 319
North	81 394	85 023
Center	81 157	44 780
Lisbon	622	3 349
Alentejo	184 157	357 799
Algarve	8 854	2 369

Alentejo is the region with the major production of table olives and olives for olive oil (Table 2). It is also in Alentjo where there is the largest production of olive oil (INE, 2017).

Table 2: Surface and production of table olives, olive oil olives and olive oil production in NUTS II (INE, 2017).

	Table olives		Olive oil olives		Olive oil
	Surface (ha)	Production (t)	Surface (ha)	Production (t)	Production (hl)
Continent	9 090	17 316	347 093	476 003	744 255
North	3 744	7 760	77 650	77 263	126 339
Center	1 534	573	79 623	44 207	65 364
Lisbon	26	8	596	3 341	0
Alentejo	3 550	8 864	180 607	348 935	549 683
Algarve	236	111	8 618	2 258	2 870

The olive groves can be classified, according to the cultural and management system, into traditional, intensive and super-intensive.

In the traditional olive grove, trees are planted with wide spacing, ranging from 60 to 200 trees per hectare. These olive groves are mainly rainfed, without irrigation systems and take about 15 years to reach production (Azeite do Alentejo, 2018). Having a higher percentage of native or local cultivars, are likely more resistant to pests and diseases, as well as more adapted to the scarcity of water that has been accentuated over the years, due to the phenomenon of climate changes (Coelho & Machado, 2016). The most commonly used olive variety is 'Galega vulgar' however, in Alentejo region, 'Carrasquenha' and 'Azeiteira' are also used, and in Beira Interior, 'Bical' and 'Cornicabra', in Trás-os-Montes, 'Madural' and 'Cobrançosa' and in Ribatejo, 'Arbequina' and 'Lentisca', are also varieties that could have significative presence (Cordeiro, 2014).

The intensive olive grove is composed by trees planted with a tighter spacing than the previous one, with the average of 285 to 415 trees per hectare and being mostly irrigated. The start of production is usually after 5 to 7 years after planting (Azeite do Alentejo, 2018). In the super-intensive olive groves, spacing can establish around 1.600 to 2.200 trees per hectare. They are usually planted in irrigated land and go into production after 3 years (OLINT, 2018). Both intensive and super-intensive regimes use varieties specially adapted to the specificities of this managements, such as Cobrançosa, Arbequina, Picual, Arbosana and Koroneiki (CAP, 2019).

2.4 Pests

The olive tree is very susceptible to the attack of several pests and diseases. These can considerably decrease production or affect the final quality of the olive to be used for olive oil and table olive production. In the Mediterranean region the main pests responsible for production damage are the olive fly (*Bactrocera olea*), the olive moth (*Prays oleae*) and the black scale (*Saissetia olea*) (Alvarado *et al*., 1999).

2.4.1 Olive fruit fly, *Bactrocera oleae* (ROSSI)

Systematic and morphology

The olive fruit fly belongs to Tephritidae family. This family of Diptera order is the most diverse, comprising nearly 4.500 described species, with some of the world most significant agricultural pests (Daane & Johnson, 2010).

The adult of the olive fruit fly is normally 4 to 5 mm long and can reach 10 to 12 mm of wingspan (Cantero, 1997; Garcia, 2000). The thorax is dark brown with 4 gray or black longitudinal bands. The scutellum is almost entirely yellow-ivory (Neuenschwander *et al*., 1986; Civantos, 1999). The wings contain dark veins and a small dark spot at each edge (Daane et al., 2004). Females can be distinguished from males by the ovipositor, a pointed structure at the end of female's abdomen (Cantero, 1997).

The eggs are elongated and cylindrical, white and very small (Neuenschwander *et al*., 1986). Its dimensions are about 0.8 mm long and 0.2 mm wide (López-Villalta, 1999), and were laid in a cavity punctured by the female in the mesocarp of the fruit, at about 1.5 mm deep, in an oblique direction (Patanita, 1995).

The larvae are apodous, cylindrical, white-yellow colored with dark mandibles. Larvae development passes through three stages, where newly larvae measure about 1 mm long (Garcia, 2000), and at the end of development can reach about 7 to 8 mm (Civantos, 1999).

Pupae have elliptical shape and their color varies from pale white to light yellow (Daane *et al*., 2004). Its dimensions are about 4 to 4.5 mm long (Arambourg, 1984; Cantero, 1997; Civantos, 1999).

Life cycle

The number of annual generations is variable, since it depends on climatic factors and varies according to the region. Thus, there are two or three generations in regions with

continental climate, depending on the summer temperature and, occasionally, three or more in the coastal areas of the Mediterranean region (López-Villalta, 1999).

Females have been reported to lay from 10 to 40 eggs per day, generally one egg in each fruit, and from 200 to 500 eggs during their lifetime (Daane *et al*., 2004).

Adults feed on a variety of organic sources including insect honeydews, plant nectar, pollen and fruit exudates, while olive fruit fly larvae are dependent on the presence of *Olea* fruit (Daane & Johnson, 2010).

B. oleae overwinters either as an adult or as pupae in the soil, 1 to 3 cm deep (Neuenschwander *et al*., 1986). New adults from overwintered pupae or first generation begin to emerge in spring, generally in April and May (Civantos, 1999), and immediately begin their activity looking for food. The lifetime of the adult is very variable, ranging from 3 to 8 weeks, depending on the temperature (Garcia, 2000).

From late June to July as new olives develop, females actively seek and oviposit in early maturing fruits (López-Villalta, 1999), usually in July in the Mediterranean region (Rice, 2000). Eggs are laid in olives under the epidermis by the ovipositor, so that the neonate larva has access to food (Torres, 2007). The egg hatching occurs over a variable period depending on weather conditions, usually eggs require a period between 12 to 19 days in early winter and only 2 to 4 days in summer (Katsoyannos, 1992).

The newly hatched larva feeds and grows in the mesocarp of olives developing a gallery inside, which becomes deeper as the larva develops, reaching the endocarp. In this gallery, the larvae reach three different instars until the end of its development. Larval stages develop from mid-summer to late autumn when there are fruits available and larval period varies from 10 to 25 days (Garcia, 2000). At the end of third instar the larva moves to the proximity of the fruit surface and pupates, with the pupa period varying greatly, ranging from 8 to 10 days, for the summer generations, and several months for the winter generations (Garcia, 2000). After pupating, the adults emerge and fly away leaving an emerging hole (Civantos, 1999), beginning a new generation. Olive flies can pupate within the host fruit during warmer months, but in late autumn/early winter, its behavior changes, and larva leaves the fruits to pupate in the ground or in any protected niche, where it remains during winter (Daane et al., 2004).

In summer, olive fly can complete a generation in 30 to 35 days, at optimum temperature, and 130 to 160 days in winter (Neuenschwander *et al*., 1986) (Figure 3).

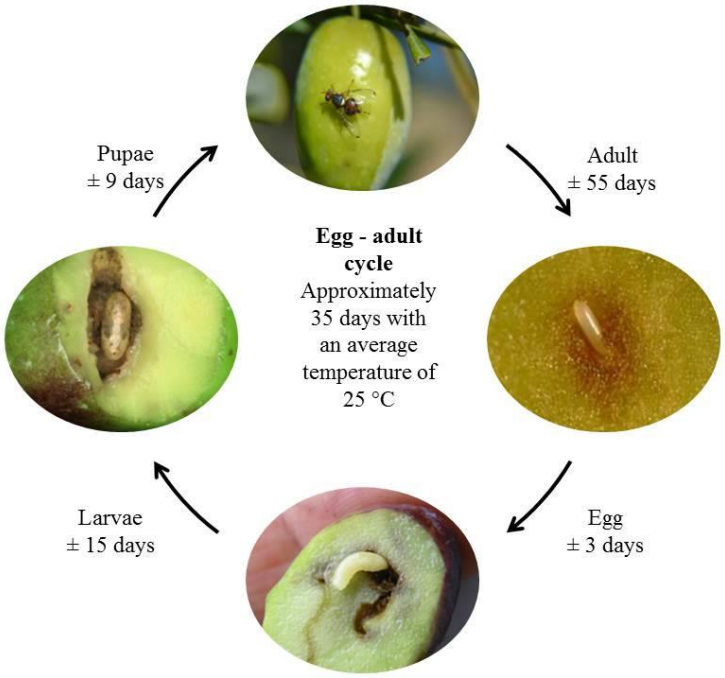

Figure 3: Life cycle of the Olive fruit fly, *Bactrocera oleae* (ROSSI)
Source: juntadeandalucia.es; agroportal.pt, 2019

Conditioning factors

Olive fly populations are subjected to natural mortality factors of variable importance depending on the time of year and environmental conditions. The temperature is one of the factors that most affects olive fly populations' abundance. Adults can survive in temperatures ranging from 6 to 35 ºC (López-Villalta, 1999), but egg laying ceases below 15 ºC or above 35 ºC (Kapatos, 1981). Eggs develop in temperatures between 5 and 37 °C, while larvae and pupae require temperatures between 6 and 30 °C. High summer temperatures associated with low relative humidity increases mortality of immature stages (Pucci *et al*., 1985). During winter, the combined action of low temperatures and high soil moisture can cause high mortality in pupae buried in the ground (Neuenschwander *et al*., 1986).

Functional diversity composed by arthropods could be responsible for constraining the population of olive fruit fly, namely parasitoids and predators. The olive fly parasitoid complex in the Mediterranean basin is thought to be relatively poor and is considered to have little effect on its populations (Torres, 2007). The most important species are usually *Eupelmus urozonus* Dalman, *Pnigalio agraules* Walker, *Eurytoma martelli* Domenichini and *Cyrtoptyx latipes* Rondani (López-Villalta, 1999). Some importance is given to predators, especially for their action on pupae. It relates to auxiliary insect families, such as carabidae, staphylinidae, forficulidae and formicidae, as well as birds and possibly small mammals (Torres, 2007).

Damages to olive crop

The importance of the damage caused by this insect varies considerably depending on the region, the years and the type of olive product. Whenever it is intended to produce table olives, the punctures carried out by this insect reduce the commercial value of the fruits and in this case the losses can reach 100% (Broumas *et al*., 2002); losses up to 80% have been reported when the production is meant for olive oil extraction (Tzanakakis, 2006). Direct damages result from pulp destruction by larvae feeding (Neuenschwander & Michelakis, 1978) and premature fruit fall to the soil (Bento *et al*., 2009).

Other indirect damages result from the adult emergence holes that could favors the penetration and attack of bacteria and fungi that decomposes the pulp (Vossen *et al*., 2004) increasing hydrolysis, oxidation, and decreasing the antioxidant compounds of oil, causing olive oil quality deterioration, and resulting in total trade devaluation in case of table olives (Civantos, 1999). This relationship is influenced by the presence of microorganisms such as bacteria (e.g. *Xanthomonas*), yeasts (mostly *Torulopsis* and *Candida*), and molds (mainly *Fusarium* and *Penicillium*), with has a positive logarithmic relationship between microflora populations and oil acidity (Torres-Villa *et al*., 2003)

Pest management measures

Indirect measures

Olive varieties vary in terms of *B. oleae* preference and studies about host preference found several factors influencing their choice for oviposition such as fruit size, colour, and epicarp hardness (Neuenschwander *et al*., 1985). Laboratory studies confirmed that Portuguese varieties, such as Cobrançosa, presents lower susceptibility to olive fruit fly when compared with others, like Madural or Verdeal Transmontana (Bento et al., 2009). The knowledge about the existence of differences in olive varieties sensitivities to olive fly attacks have great interest at the time of installation of the olive grove, especially in areas of greatest risk of attack (Gomes & Cavaco, 2003).

With the objective to enhance the control exerted by natural enemies in the olive groves, attention has been both dedicated to increase plant diversity associated with ecosystem and to implement within the crop, artificial foods resources (Torres, 2007). In olive groves, the use of ecological infrastructures can have an important role in improving and conserving biodiversity (Serrano, 2016). The implementation of spontaneous vegetation in olive groves is considered particularly interesting because it can provide shelter, food and can be a reservoir of alternative prey species for predators and parasitoids (Campos & Civantos, 2000).

Usually, due to extreme summer hot temperatures, olive fly can only be active in the autumn months, where associated damage is most severe, although this effect can be reduced by harvest anticipation. Attacked olives fallen to the ground, as well as buried pupae that spend the winter in the soil, are hotspots of olive infestation and should be eliminated, especially in years of severe pest attack. In this case, fruits should be picked and removed, it is advised to perform superficial mobilizations under the canopy after harvest, to expose the pupae to adverse weather conditions and entomophagous action (Torres, 2007).

Direct measures

In the field of biological control through entomophagous arthropods, efforts to incorporate biological control in *B. oleae* management were initially made using the braconid wasp, *Psyttalia concolor* (Hoelmer *et al*., 2011), which was introduced into Italy from Tunisia in 1914 and later in other Mediterranean countries. This parasitoid was repeatedly introduced but it did not establish widely in Europe, which was attributed to unsuitable climatic conditions (Miranda *et al*., 2008) and due to the lack of synchronism between the parasitoid and its host (Clausen, 1978), resulting in low rates of parasitism (Jiménez et al., 1990). The use of *P. concolor* releases seems to have failed to control *B. oleae* populations (Delrio et al., 2005). Also in biological control, there is a great interest currently focused on spinosad, a naturally derived insecticide produced by fermentation of the actinomycete *Saccharopolyspora spinosa* Mertz & Yao (Thompson *et al*., 2000). The exposure to spinosad results in feeding inhibition followed by involuntary muscle contractions, prostration with tremors, paralysis and eventually, death (Salgado, 1998). The spinosad was introduced in 1997 and since then there have been several cases of resistance to spinosad in field populations of insect pest species, in other cultures than olive orchards, which have led to reduced efficacy. Overuse or misuse of any new insecticide product such as spinosad, can lead to the development of resistance. Therefore, the use of label restrictions and guidelines designed to minimize the chances of resistance development is especially important for an insecticide like Spinosad that has been registered for use on a wide range of pests and crops (Sparks *et al*., 2012)**.**

In the field of biotechnical control the recognition of the fact that olive fly responds strongly to food, visual and sexual stimuli has encouraged the development of controlling strategies that take advantage of this response, such as mass capture, whose goal is to attract and capture/or kill large amounts of insects (Torres, 2007; Torres *et al*., 2009).

Finally, chemical control against olive flies are traditionally achieved by the use of organophosphate insecticides in cover and/or bait sprays (e.g., dimethoate and fenthion) (Daane & Johnson, 2010), and according to two modalities, one targeting adults and the other focuses on larvae. The first of these modalities is preventive and aims to eliminate adults before the oviposition. This objective could be achieved using an insecticide combined with an attraction, generally of food nature, thus reducing the sprayed area. The

second modality, curative, against adults and larvae, involves spraying the entire canopies of the olive orchards (López-Villata, 1999).

The extensive and long use of insecticides for the control of *B. oleae*, apart from the adverse side effects on beneficial organisms, might lead to the development of insecticide resistance, especially when only one group of insecticides with a particular mode of action is used constantly (Skouras, 2007). Resistance to organophosphates is known since the 60s and has increased drastically since then, being widespread in the Mediterranean region (e.g. Lantero *et al.*, 2020; Nobre *et al*., 2019; Pereira-Castro *et al*.; Vontas *et al*., 2011).

2.4.2 Olive moth, *Prays oleae* (BERNARD)

Systematic and morphology

Prays oleae belongs to Yponomeutoidea superfamily, which has been subjected to several modifications in the last few years. Some previous subfamilies of Yponomeutidae were separated in independent families following results from molecular studies (Nieukerken *et al*., 2011) and Praydidae is now considered a family, including 51 species, where *P. oleae* was included.

Species variability has been encountered that poses the question on the existence of cryptic species, but lineage-specific differences in biological traits were not yet demonstrated (Nobre *et al*., 2018). Such traits, if existing, can have impact on the behavior of the potential pest and severity of its activity.

The adult is a small lepidopteran, of silvery gray color, measuring about 6 to 6.5 mm in length, and 13 to 16 mm of wingspan (Cantero, 1997). In males the abdomen is thin and ends abruptly, in females it is bulkier, and pointed, and covered with very long fine hairs (Garcia, 2000).

The *P. oleae* egg is milky white and has an oval shape, with about 0.5 mm in length (Arambourg & Pralavorio, 1983).

The size of the larva varies from 0.6 mm at birth to 7 mm in lenght when it reaches its maximum development, going through five larval stages. It has a yellowish-white coloration where the brownish color of the head is highlighted (Civantos, 1999).

The pupa is wrapped in a silk cocoon of white color and very loose mesh. Initially light green, becoming brownish and finally acquiring a grayish color, signaling that the period of adult emergence approaches. The pupa measures about 5 to 5.5 mm in length (Garcia, 2000).

Life cycle

The olive moth is a monophagous species, which has three annual generations, each one developing at the expense of a different organ from its host, such as leaves (phylophagous generation), flowers (antophagous generation) and fruits (carpophagous generation) (Torres, 2007).

The phylophagous generation (first generation) occurs during late autumn and early spring resulting from eggs laid between mid-September and early October, by the adults belonging the previous generation (carpophagous), or until early November according to Alvarado *et al*. (1999). The females oviposit usually on the upper page of the leaf, next to the central vein (Guerrero, 1991), and under natural conditions the egg incubation lasts 7 to 16 days (Pelekassis, 1962).

After the egg hatching, the larvae immediately puncture the leaf epidermis, reaching the parenchyma, from which they feed for the first four instars, reaching the fifth instar to feed on the outer part of the leaves and even young shoots (Arambourg & Pralavorio, 1983). According to Cantero (1997) the larvae make a characteristic C-shaped, subcircular or circular galleries which are easily recognized.

When the larva finishes its development, it stops feeding and weaves a cocoon, between two overlapping leaves or next to a shoot, during the second week of March and the

first of April, so that the first adults appear from the beginning of April after it pupates for a period of 15 days (Alvarado *et al*., 1999; Alvim, 1963).

The emergence of the moths usually occurs at night or in the morning, during the month of April (Azevedo, 1965). *P. oleae* adults manifest a negative phototropism, remaining motionless under the leaves during the day and flying at dusk (Arambourg, 1964).

The duration of the phyllophagous generation is between 180 and 230 to 260 days (Arambourg, 1964) (Figure 4).

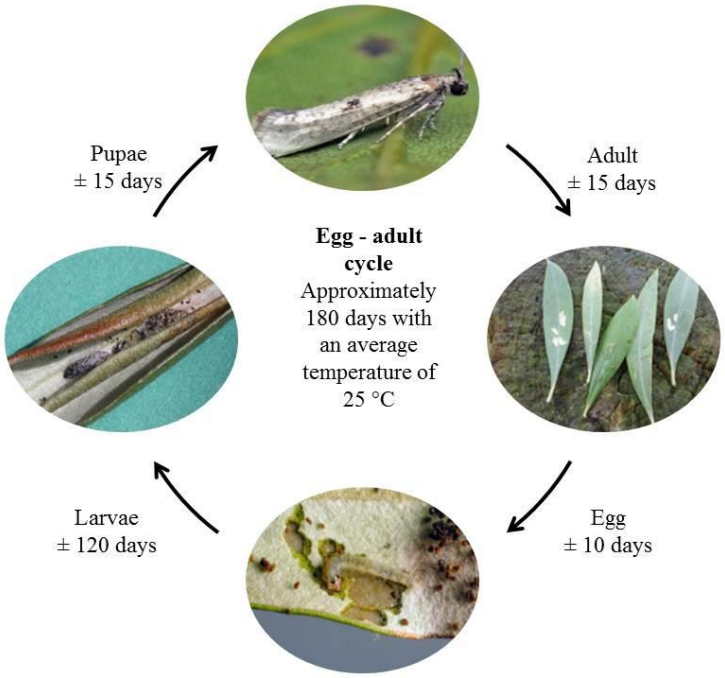

Figure 4: Phylophagous generation of the Olive moth, *Prays oleae* (BERNARD)
Source: agrochem.es; agroes.es 2019

The antophagous generation (second generation) starts with adults from the previous generation (phylophagous) ovipositing in the flower buds as soon as they are receptive. Alvim (1963) refers that oviposition starts in late March until the end of April, and the incubation period is about a week or less, influenced by temperature, or 10 to 12 days according to Pelekassis (1962) and Arambourg (1964).

After hatching, the larvae feed initially on the anthers; later consume the stigma, stiletto and ovary, eventually destroying the entire flower. The symptoms are easily

detectable by the existence of silky threads in which excrements and remains of brownish petals accumulate involving the buds (Torres *et al*., 2003), and which eventually will compromise the fruit set (Azevedo, 1965).

Larval development is fast and takes about 20 to 35 days, due to the quality of food available and favorable climate conditions (Arambourg, 1985). The larva weaves a cocoon into the destroyed flower buds and pupates. The duration of the pupal phase is, approximately one week (Pelekassis, 1962; Alvim, 1963; Cantero, 1997), and 15 days according to Arambourg (1966). As reported by Alvim (1963), this generation starts ovipositing at the end of March until the end of April and the adults appear in late May and early June, in Portugal (Bento *et. al*., 2005).

The antophagous generation presents the shortest duration, averaging between 45 and 55 days, according to Arambourg (1964) and Pelakassis (1962) (Figure 5).

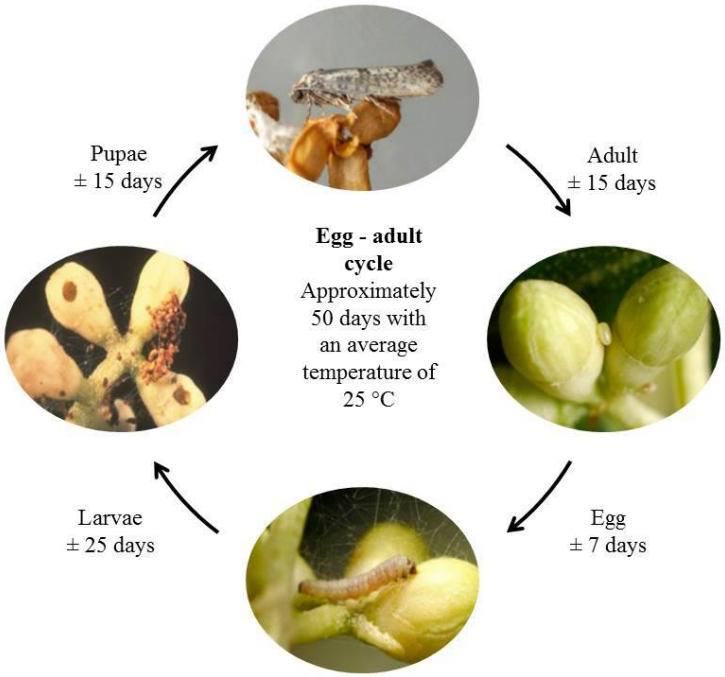

Figure 5: Antophagous generation of the Olive moth, *Prays oleae* (BERNARD)
Source: juntadeandalucia.es, 2019

The carpophagous generation (third generation) begins with the oviposition on young fruits, preferably on the calyx, 90% of cases are near the peduncle insertion (Alvarado,

1964). However, they can also be found in other parts of the fruit in case of strong attacks or when fruiting is scarce (Alvim, 1963). The oviposition takes place in May, lasting until early July (Alvim, 1963) and the egg incubation period varies from 6 to 7 days (Arambourg & Pralavorio, 1981).

Normally all larvae reach the fruit, but only some proceed their development, inside the kernel, which varies between 80 and 150 days (Arambourg, 1964). Pupation usually occurs in the ground, especially if the larvae had no time to abandon the fruit before its fall, otherwise it pupates on leaves or trunk (Arambourg, 1964).

Adults of the carpophagous generation appear is in mid or late September (Bento *et. al*., 2005), and the average duration of the generation is between 90 and 163 days (Arambourg, 1964) (Figure 6).

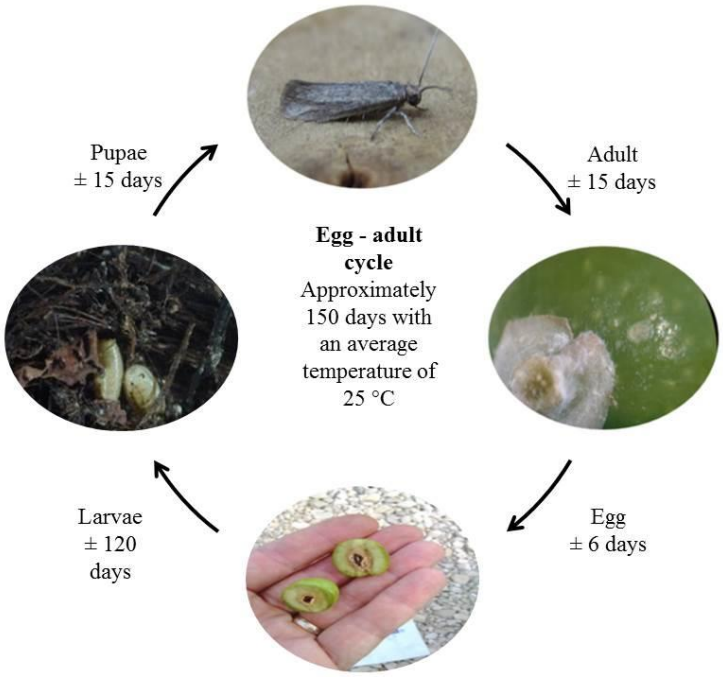

Figure 6: Carpophagous generation of the Olive moth, *Prays oleae* (BERNARD)
Source: juntadeandalucia.es, 2019

Under natural conditions, the adult longevity of *P. oleae* is on average 15 days (Arambourg & Pralavorio 1983).

Conditioning factors

Temperature and relative humidity play a key role in the regulation of *P. oleae* populations and are largely responsible for the differences in attack intensity of successive generations and between regions (Torres, 2007).Therefore, at temperatures above 35 °C, associated with relative humidity below 50%, almost all eggs can be damaged (Arambourg & Pralavorio, 1983; Arambourg, 1985).

Young larvae are also particularly affected by abnormally high or low temperatures. Larvae of the phylophagous generation develop slowly due to the low temperatures that occur in winter, which can cause high mortality, especially when they occur in February, period in which they leave the galleries (Arambourg, 1964). Larvae of the carpophagous generation have difficulty penetrating the fruit and are destroyed by temperatures above 30 °C and relative humidity around 20% (Arambourg & Pralavorio, 1986). Pupae only suffer mortality at temperatures above 40 °C and with relative humidity below 60% (Arambourg, 1985).

The adult activity decreases when temperatures are below 12 °C, they may even be harmful when they drop below 7 °C.

The action of predators and parasitoids is undoubtedly one of the most important factors for regulating *P. oleae* populations. López-Villalta (1999) indicates as predators different species of spiders, which feeds on *P. oleae* eggs and larvae. With special interest the author mentions the neuroptera *Chrysoperla carnea* Stephens, since it is very effective on natural control of insects, feeding with great avidity on eggs, larvae and pupa. According to Arambourg & Pralavorio (1983), predation rates on eggs can reach values between 80% and 90%. Other species also mentioned, although of less importance, are sirphids, mites, ants and coccinellids (Torres, 2007).

The parasitic complex of *P. oleae* includes both polyphagous and specific species. Among these auxiliaries, standing out for the abundance with which they had been observed, are the hymenopteran of the families' Braconidae, Chalcididae, Eulophidae, Ichneumonidae and Trichogrammatidae. The following species have special importance since they are

specific to *P. oleae*, and/or due to their frequency in the Mediterranean region, being: the encyrtid *Ageniaspis fuscicollis* Dalman, the braconids *Chelonus elaeaphilus* Silvestri and *Apanteles xanthostigma* Haliday, the eulofids *Pnigalio agraules* Walker and *Elasmus flabellatus* Fonscolombe, the ichneumonid *Diadegma armillatum* Gravenhorst and species of the genus Trichogramma (Torres, 2007).

Damages

The damage caused by *P. oleae* can be classified into three types, each one associated to a generation (Patanita, 1995):

a) The terminal buds destroyed by the larvae of the first generation, preventing the normal growth of trees and compromising its further development. This type of damage is only considered important in young olive groves;

b) In flowers, the second generation can cause the destruction of important parts of the inflorescences, which can have an impact on production;

c) In olives, the galleries made by the larvae of the third genteration cause the fruit to fall, both in early summer, at the time of larva entry, as in late summer, when the larva is leaving the fruit completely developed.

Pest management measures

Indirect measures

The conservation of indigenous arthropodofauna is considered particularly important in the case of olive moth, given the richness and diversity of its predators and parasitoids species. In this sense, it is advisable to encourage the growth and stablishment of their

populations by providing them alternative hosts, supplementary food, shelters and hibernation sites. More interesting should be the use of natural vegetation, which is known to include plants that favor the action of the auxiliary fauna (Torres, 2007).

Among the cultural techniques, the most important in limiting the population of the olive moth is undoubtedly pruning. By suppressing 40 to 50 % of the tree leaves, it is possible to eliminate about 25% of the pest population, especially when carried out at the period when the larvae and eventually pupae of the phylophagous generation develop (Arambourg & Pralavorio, 1983). According to the same authors, this technique, combined with the natural fall of leaves, may be responsible for a reduction of the pest population that can reach 40%.

Direct measures

In the field of biological control, the currently use of entomophagous insects to control *P. oleae* has proved ineffective and economically unacceptable. However, it is thought that the use of the egg parasitoid trichrogramma in biological control with successive releases may have some success (Patanita, 2007). The interest in these auxiliaries is their ease rearing, and it has already been tested in Trás-os-Montes, against the carpophagous generation of the moth, with very promising results (Alcobia & Ribeiro, 2001).

Regarding microbiological control, the use of *Bacillus thuringiensis* for controlling the antophagous generation has proved to be effective. *B. thuringiensis* is an entomopathogenic bacterium that produces specific toxins which acts on the larvae digestive tract. In consequence, within a few minutes to 2 hours after ingestion the larva stop feeding, dying after 2 to 5 days (Regato, 2007). In addition to the advantage of its specific action, this entomopathogenic bacterium does not normally present toxicity to humans, domestic animals, pollinator insects and natural auxiliaries (Amaro & Baggiolini, 1982).

As for the chemical control, phytosanitary treatments are currently directed to the larvae, wich are directly responsible for the losses, in particular in the first and second

generations. Interventions against anthophagous generation should be performed when most larvae are in the third instar, usually at the beginning of flowering, using organophosphate insecticides (Civantos, 1986).

2.4.3 Black scale, *Saissetia oleae* (OLIVIER)

Systematic and morphology

The black scale, *S. oleae* is a polyphagous species, which the main hosts are olive trees and citrus (Passos-Carvalho *et al*., 2003). It belongs to the order Hemiptera and to the Coccidae family. It is an oviparous species with parthenogenetic reproduction, in which males are very rare and unknown in Europe (Cantero 1997).

The female goes through three instars before reaching adulthood (Torres, 2007). The egg is protected by the female body shield, measuring approximately 0.27 mm of major axis and 0.14 mm of smaller axis, thus it presents an ellipsoid shape (Passos-Carvalho *et al*., 2003). At first, it has a pale-pinkish coloration becoming darker close to hatching. Its incubation lasts from 5 to 20 days in the spring (Garcia, 2000).

In the first instar, the nymph is light yellow in color and its length is less than 0.5 mm. In the second stage, the nymph has an orange color; length between 0.5 to 0.8 mm and an "H" shaped appears in relief in the back. The third stage nymph has a dark color and measures 0.8 to1.5 mm (Montiel & Civantos, 1991).

The fourth instar nymph or young female adult, measures 2 to 5 mm in length, 1 to 4 mm in width (Passos-Carvalho *et al*., 2003). At this stage, their mobility is much reduced, they travel short distances, which can lead to compact concentrations of *S. oleae*, with an overlap of the margin part of the body with other scales, resulting in a deformed body due to the competition for space (Passos-Carvalho *et al*., 2003).

When the convexity of the dorsal region of the body is accentuated the period of laying eggs approaches (Passos-Carvalho *et al*., 2003). At this stage an egg chamber is

formed, and the scale becomes darker (Pereira, 2004). The scale shows some preference in fixing itself to the branches of the host due to the greater abundance of the sap, compared to the leaves. Their mouth parts are more developed than in nymph stage, which allows them to feed deeper in hard and stiff surfaces (Pereira, 2004).

Life cycle

In olive trees, the black scale usually develops an annual generation. However, in certain situations it can complete two generations. Among the reasons for the second generation are particularly the conditions of higher humidity and climatic mildness of coastal regions and the improvement of the nutritional conditions of the host plant conditioned by cultural practices such as nitrogen fertilizers and irrigation. Another assumption is that extreme temperature tolerant genotypes may occur in some populations (Torres, 2007).

The black scale hibernates in the form of nymphs of second and third instar. Adults appear from May to July and the eggs are laid from June to August (Coutinho, 2011). The incubation period lasts an average of 10 to 15 days, in the spring and early summer and 20 to 25 days in autumn (Passos-Carvalho *et al*., 2003). These eggs hatch and the nymphs may appear from June to September. In more favorable regions or years, early nymphs develop rapidly and give rise to a second generation (Pereira, 2006). The newly hatched nymphs of the first instar remain in the egg chamber for one or two days (Torres, 2007), after the larvae move out of the egg chamber beneath the mother's body they wander over the host plant searching for a suitable place to settle. The first instar lasts about four weeks in early summer (Morillo, 1977), and up to 50 days in late autumn (Torres, 2007). They generally prefer to colonize a suitable place nearest to the mother scale, consequently, they tend to form groups and their distribution on the host plant is highly aggregated (Briales & Campos 1986).

The second instar lasts between 10 to 15 days in the summer up to 70 days in late autumn and the duration of the third instar is longer and varies between two and four weeks

for individuals who develop in the summer, and seven weeks for those who hibernate (Passos-Carvalho *et al*., 2003).

The longevity of adult females varies widely, not only between individuals but also according to the period of the year in which the insect develops, being approximately two months for individuals developing in early summer and more than four months, for those that develop in late autumn (Torres, 2007). It is in the period that preceds the egg laying phase of *S. oleae*, that the attack on the host assumes greater severity, due to intense food and excretory activity. When the female initiate oviposition it stops feeding and its resistance to chemicals is greater. At the end of this phase, the female enters in decrepitude, dies and dries, but remains fixed; the dry shell protect the eggs, but can also be used by other organisms as a shelter (Cabanas, 1998) (Figure 7).

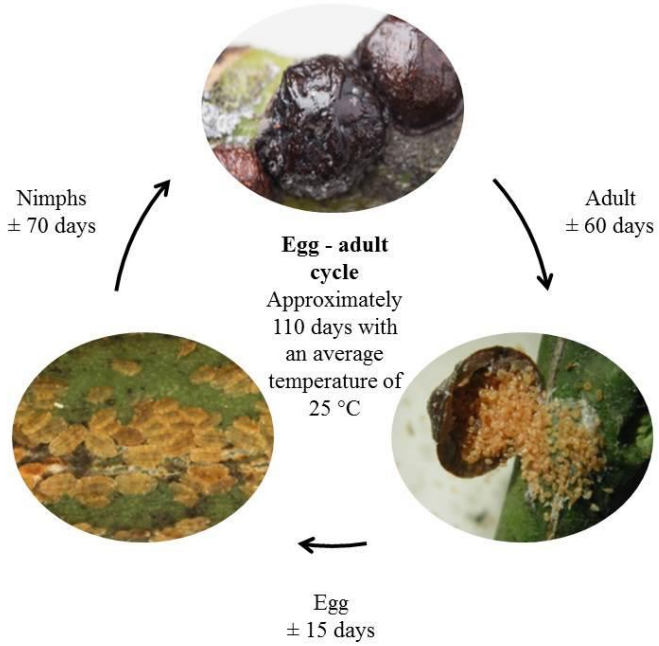

Figure 7: Life cycle of the Black scale, *Saissetia oleae* (OLIVIER)
Source: upv.es, 2019

Conditioning Factors

Among the factors that influence the presence of the black scale, temperature, relative humidity, tree vigor, and the presence and abundance of auxiliaries are predominants (Torres, 2007).

Temperatures that favor the development are those with maximum values between 22 and 30 ºC and minimum values between 10 and 14 ºC (Torres, 2007). Moreover, according to Civantos (1999), temperatures higher than 35 °C, associated with low relative humidity, can cause mortality levels in newly hatched nymphs higher than 90%.

The wind has been also pointed out as being able to exert a mechanical action on the first instar larvae, on its mobile period, contributing to its dispersal and colonization to neighboring hosts (Pereira, 2004). Tight spacing that hinders air circulation, inadequate pruning, excessive nitrogen fertilization and irrigation are conditions which favor dense canopies, and subsequently the development of the pest (López-Villalta, 1999).

As for the complex of natural enemies associated with the black scale, the role of both entomophagous predators and parasitoids is noteworthy and several species are known (Pereira, 2004). Referred parasitoids belong to the order Hymenoptera, and are distributed among three families (e.g. Aphelinidae, Encyrtidae and Pteromalidae), regarding the predators those belonging to the coccinelidae family are mainly the most present and effective (Santos, *et al*., 2008b). According to López-Villalta, 1999, under normal conditions the action of these entomophagous insects, particularly the parasitoids, is sufficient to maintain pest populations at a tolerable level.

Damages

The black scale attacks are easy to identify by the presence of the insect on the branches, leaves, and more rarely on the fruits, which is often associated with the development of the fungus complex commonly referred by ‘fumagine’, giving those organs a blackened appearance (Torres, 2007).

Plant damages can be of a direct or indirect nature. The direct damage is related to the insect's feed process and it is caused by the sap feeding, which eventually weakens the plant (Torres, 2007). These damages are generally of little economic importance in adult trees, since toxic effects resulting from their feeding activity are not evident. In very young trees, their presence and feeding action can negatively affect the future tree growth. The indirect damages are those that come from the excreting of honey dew by the black scale, and favors the development of 'fumagine', which is a complex of saprophytic fungi, that cover the surfaces of the leaves (Santos *et al*., 2008), and may cause physiological alterations, particularly in photosynthetic, respiratory and transpiratory activity (Passos-Carvalho *et al*., 2003). As a consequence, defoliation can occur with the depletion of the branches and the decline of the vegetative state of the plants, leading to a reduction of production, and in extreme situations lead to total loss (López-Villalta, 1999). Defoliation is a serious condition is especially evident on young trees.

Pest management measures

Indirect measures

The adoption of balanced cultural practices plays an important role in the olive grove. Thus, the crop system management should allow optimizing factors such as aeration and light penetration. In particular, pruning must enable adequate illumination and air circulation in the canopy. Watering and fertilization, particularly nitrogen, should be applied according to the needs of the crop, not promoting excessive vigor of the trees (Torres, 2007).

The protection, maintenance and increase of auxiliary population is of interest. It should be noted that the entomophagous auxiliar complex associated with black scale insects is relatively rich, including a few dozen parasitoids and predators. Among the first are the encyrtid *Metaphycus lounsburyi* Howard, *M. flavus* Howard and *M. helvolus* Compere, the pteromalid *Scutellista caerulea* Fonscolombe and *S. obscura* Förster, and the aphelinid of the genus Coccophagus, such as *C. lycimnia* Walker and *C. semicircularis* Förster, all

present in Portuguese olive groves (Torres, 2007). Regarding the predators, some species of the coccinellid family, have been pointed out as having great importance in the natural limitation of black scale insects. Also, entomopathogenic fungi, such as *Verticillium lecanii* (Zimm.) Viegas and *Fusarium larvarum* Fuke, have been shown to be able to attack different insect developmental stages (Passos-Carvalho *et al*., 2003).

Direct measures

Biological control against black scale has a long tradition, and many initiatives were carried out within it. Thus, in the 1890s and early 1900s, it was put into practice in California, one of the largest biological control campaigns ever undertaken against this pest, with the introduction of 40 exotic species of parasitoids imported from Africa, Asia , Australia, Central and South America, Europe and the Middle East (Bartlett, 1978). In the following years, intense activity was developed in this field, with the introduction of exotic auxiliary species in several Mediterranean countries, such as Israel, France, Greece, Italy, Spain and Portugal. In general, these studies focused on parasitoids of the species *Metaphycus swirskii* Annecke & Mynhardt, *M. lounsburyi* Howard, *M. helvolus* Compere and *Diversinervus elegans* Silvestri. Although obtained results have been variable, it is now consensual that biological control can contribute effectively to the protection of olive groves against the black scale (Torres, 2007)

Concerning the chemical control summer oil may be used, taken into account all the recommendations regarding its use, in particular should not to be sprayed during the flowering stage. To be effective it should be directed to the young forms of the insect (first nymph stage), immediately after the outbreak of the majority of the population, which usually corresponds to the month of July (Alcobia & Ribeiro, 2001).

2.5 Natural limitation of pest populations

Biodiversity performs a variety of ecological services including recycling of nutrients, regulation of microclimate and local hydrological processes, detoxification of noxious chemicals, suppression of undesirable organisms, etc. (Koohafkan *et al*., 2011). In this last category we can include biodiversity role in aiding controlling pest population outbreaks, which encompasses the promotion of biological protection through native auxiliary fauna (Martínez-Ferrer *et al*., 2020).

Pest control by natural enemies arises as an ecologically and economically promising solution. Among natural enemies, both predatory and parasitic insects have been shown to be effective in suppressing pest species (Dainese *et al*., 2017). Many natural enemy populations possess behavioural adaptations that are required to maintain pest populations at non-economic densities. Some of these are: they should coexist in time and space, possess a high reproductive response to slight increases in host density, and show seasonal reproductivity equal to or greater than that of the pest population (White, 2019). However, to actually be able to assign a role in suppressing pest population size, several knowledge gaps need to be fulfilled (Figure 8).

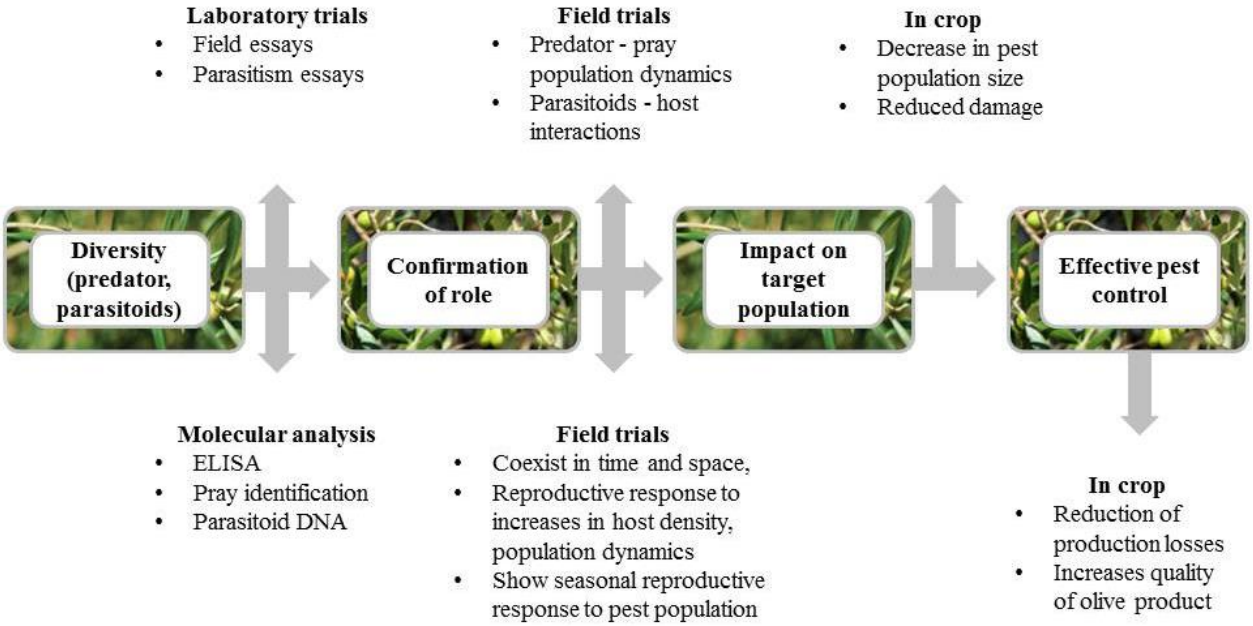

Figure 8: Schematic representation of research steps needed to relate auxiliar fauna and pest management. The final aim is a reduction of production losses and an increase of olive products quality (Martínez-Ferrer *et al*., 2020).

Unarguably, the first step is a biodiversity assessment, which for the aim of management of the putative target pests should focus on the entomological diversity and on the main guilds of natural enemies: predators and parasitoids. However, their presence does not indicate *per se* that: 1) they feed or parasitize the desired host; 2) their ecological dynamics matches the one of the targeted putative pests; and 3) that increase rates of parasitism/predation cause a reduction of pest population. The final proof-of-concept is a measurable decrease in crop damage that can be attributed to the action of this auxiliary fauna (Martínez-Ferrer *et al*., 2020).

As a first step the correct identification of the species present is necessary. Predators, although difficult, are considered an easier group for morphological identification (dominated by ants and spiders). Parasitoids, however, are recognized as highly challenging due to their diversity and size. Nowadays, the use of molecular tools can aid on this step and offer a new ability to identify species, albeit not without some of the same caveats for morphological discrimination (Heraty, 2017). A variety of molecular markers are available for diagnosing all levels of divergence in insects. Comparative nucleotide sequences are currently the most common choice for species recognition, identification, and phylogenetic analysis (Heraty, 2017).

2.6 Ecological determinants of parasitoid abundance and diversity

In addition to the biological interactions (biotic factors, e.g., presence of competitors and predators, quality and quantity of resources), abiotic factors, such as temperature and rainfall, and the plant selected as host are known to affect insect population dynamics (Marchioro & Foerster, 2016). Understanding environmental variability and the ways in which organisms' response over short and long timescales is of considerable importance to the field of ecology and conservation biology (Chown & Terblanche, 2007) and is a practical concern with regard to parasitoids which are key components of terrestrial ecosystems due to their diversity, abundance and functions.

Changes in temperatures have significant consequences on the phenology of parasitoids, life history as well as distribution and synchronism with their host species, which will ultimately impact the severity and timing of pest outbreaks and ecosystem functioning (Hance *et al*., 2007). Like other insects, parasitoids have body temperatures that largely track the temperature of their environment, and ambient temperatures are thus critical in determining parasitoid population dynamics and the distribution of suitable habitats. Furthermore, parasitism rates depend on the ability of parasitoids to successfully locate, select, and oviposit in, on or near their hosts (Jeffs & Lewis, 2013).

External disturbances such as drought periods, extreme precipitation and heatwave events may affect parasitoids physiological capacity to perceive chemical and visual signals from their environment (Colazza & Wajnberg, 2013). In mainland Portugal, a heatwave is characterized as an interval of at least 6 consecutive days with the maximum daily temperature 5 ºC higher than the average daily value in the reference period (IPMA, 2017). Such heatwave could have serious detrimental effects on survival, fitness, and foraging behavior of these natural enemies (Chen *et al*., 2018), thus translating into population level consequences.

Landscape complexity and how natural enemies' populations interact with it have been shown affecting both diversity and abundance of parasitoids as well (Thies *et al*., 2003; Bianchi *et al*., 2006; Rusch *et al*., 2010), due to its dependence on the plant species composition of the surrounding vegetation, and also on the spatial extent of its influence on natural enemy abundance, which is determined by the distance to which natural enemies disperse into the crop (Nicholls *et al*., 2001).

More generally, a better comprehension of the processes governing insect dynamics is needed in order to predict the consequences of changes on species interactions and synchrony across multiple trophic levels, community functioning, and ecosystem services (such as biological pest control) (Tougeron *et al*., 2020)

2.7 Molecular tools for identification of hymenoptera parasitoids

Groups of insects present a great challenge to the taxonomic work simply because of their diversity. The recognition of species by traditional morphological methods is complex and usually requires specialist knowledge, thus, the number of undescribed insect species far exceed the number of taxonomic specialists, a workforce in decline (Godfray, 2002).

Therefore, the accurate taxonomic identification is the main issue in biological research, in order to allow the implementation of adequate measures to control species of agricultural importance (Miller & Rossman, 1995). To determine the identity of parasitoids linked to a host species in different habitats and locations is relevant to understanding both ecological and evolutionary relationships between hosts and parasitoids, and to assess biological control potential of pest hosts (Tilmon *et al*., 2000).

Because of their life-strategies, parasitoids constitute a key component of nearly all terrestrial ecosystems, contributing to the regulation of arthropod populations. Despite their ecological and economic importance, relatively little is known about their diversity, distribution and biology. Their study is challenged by their typical small size, high number of species, the complexity of their life cycle and the difficulties in their taxonomy because of slight morphological differences between species (Santos *et al*., 2011).

Therefore, genomic approaches to taxon diagnosis exploit diversity among DNA sequences to identify organisms and represent one extremely promising approach to the diagnosis of biological diversity (Wilson *et al*., 2017). The identification of insects based on specific fragments of deoxyribonucleic acid (DNA) can be performed with immature insects or fragments of puparium and adult insects, and provide a faster identification (Harvey *et al*., 2003). According to Amendt *et al.* (2004) polymerase chain reaction (PCR) amplification of suitable regions of the genome, sequence analysis of the amplicons obtained, and alignment of the data with reference sequences is the usual and recommended method.

2.7.1 DNA barcoding

In the search for a simple method to identify and compare species, Hebert *et al*. (2003a) proposed the DNA barcoding, a new system of species identification using the

cytochrome c oxidase subunit 1 mitochondrial gene (cox1 or COI) as a genomic segments as markers for species recognition (Wilson *et al*., 2017).

Just as species differ in morphology, ecology, and behavior, they also differ in their DNA sequences. Hence, at least in principle, a particular gene or gene fragment can be used to recognize a given species in much the same way that retail barcodes can be used to uniquely recognize each consumer product (Wilson *et al*., 2017).

Species identification by DNA barcoding is a sequencing-based technology. Once obtained the sequence information of the target specimen it is possible to compare this information to a sequence library from known species, such as Basic Local Alignment Search Tool (BLAST), in conjunction with DNA databases such as GenBank (Floyd *et al*., 2009).

DNA extracts from any life stage of an organism or from tissue fragments will generate a similar identification, whereas traditional identification keys often depend on adult features (Wilson *et al*., 2017), such as genitalia. The key point for any taxonomic system is its ability to deliver accurate species identification and, according to Hebert *et al.* (2003a), DNA barcoding accurately identify species in more than 95% of cases.

2.7.2 Mitocondrial DNA

The particular genomic region used as a DNA barcode represents an important choice. It must be homologous between the organisms compared and have a rate of evolution fast enough to show variation between closely related species. It must have sufficient regions of sequence conservation to allow a limited set of PCR primers to amplify the target gene region from broad sections of the tree of life, and the resultant sequence information also must generate a robust alignment so that sequences can be compared (Wilson *et al*., 2017).

Generally, the mitochondrial genome (mtDNA) of animals is a better target for analysis than the nuclear genome because of its high copy number, lack of introns, its limited exposure to recombination and its haploid mode of inheritance (Hebert *et al.*, 2003b)

and therefore, have an increased chance of generating species-specific markers (Harvey *et al.*, 2003).

In animals, mtDNA occurs as a single double-helical circular molecule containing 13 protein-coding genes, 2 ribosomal genes, a non-protein coding control region, and several transference RNAs. Each mitochondrion contains several such circular molecules and, therefore, several complete sets of mitochondrial genes. Furthermore, each cell has several mitochondria. Thus, when sample tissue is limited, the mitochondrion offers a relatively abundant source of DNA (Waugh, 2007).

2.7.3 Cytochrome c oxidase subunit I (COI) as DNA barcoding marker

In the animal kingdom, attention has focused on a small DNA fragment from a standardized region of the genome (Hebert *et al*. 2003b). This fragment consists of a 658 bp string corresponding to nucleotide positions 1490-2198 from the 5'– end of cytochrome c oxidase subunit I gene (COI) using *Drosophila yakuba* mitochondrial genome as a reference (Miller & Rossman, 1995).

Hebert *et al*. (2003b) says that COI have two important advantages: (1) the universal primers for this gene are very robust, enabling recovery of its 5'end from representatives of most, if not all, animal phyla and (2) COI appears to possess a greater range of phylogenetic signal than any other mitochondrial gene (the evolution of this gene is rapid enough to allow the discrimination of not only closely allied species, but also phylogeographic groups within a single species).

After sequencing, an unknown insect sequence can be compared with a library of barcode reference sequences obtained from specimens of known identity. If it matches with a high confidence level with a reference sequence, it can be assumed that the unknown specimen belongs to the reference taxon (species) or, at least, to the group with identical species. On the other hand, if the unknown sequence does not match with any within the database, new data can be recorded as a new haplotype or a geographical variant (and in some cases, can be the unveiling of a new species) (Karthika *et al*., 2016).

3. Objectives

The adoption of practices that protect and promote biodiversity in olive groves is essential. In order to promote biological protection through conservation, the native auxiliary fauna may play an important role in maintaining olive trees pests' populations at acceptable levels.

Samplings of the entomological diversity associated with olive trees in the Alentejo region was performed, and this project aims to make an extensive characterization of sympatric putative parasitoids, in the fall period, when *B. oleae* is usually very active on olive orchards.

Within this context, the work consists on:

a) Recognition, description and screening of the different insect parasitoids sampled into morphotypes, and its likely taxonomic identity.
b) Make inferences on their relevance in the ecosystem, based on abundance, diversity, specificities of the trophic guild and taxonomic position.
c) Analyze the ecological variables and their influence on parasitoids population.
d) Amplify the nucleotide sequences of the mitochondrial cytochrome oxidase I gene (COI) towards an integrative taxonomy of each specimen.
e) Combine morphological, taxonomic, molecular and ecological data to raise hypotheses on which parasitoids present in the fall are more likely to impact on the olive fly population and in other olive tree pests.

4. Material and methods

4.1 Study area and Sampling

For the survey, a stratified random sampling was designed to cover the region. Grids of 30 x 30 km comprised the stratification of the sampling and inside each square 7 olive areas were selected. In all cases, sampled trees were taken from crops undergoing biological

production regimes or in decorative specimens, to guarantee that no pesticide had been applied directly in recent years. Therefore, each sample received a specific code, namely its collection location, including the square and the olive grove area (Figure 9).

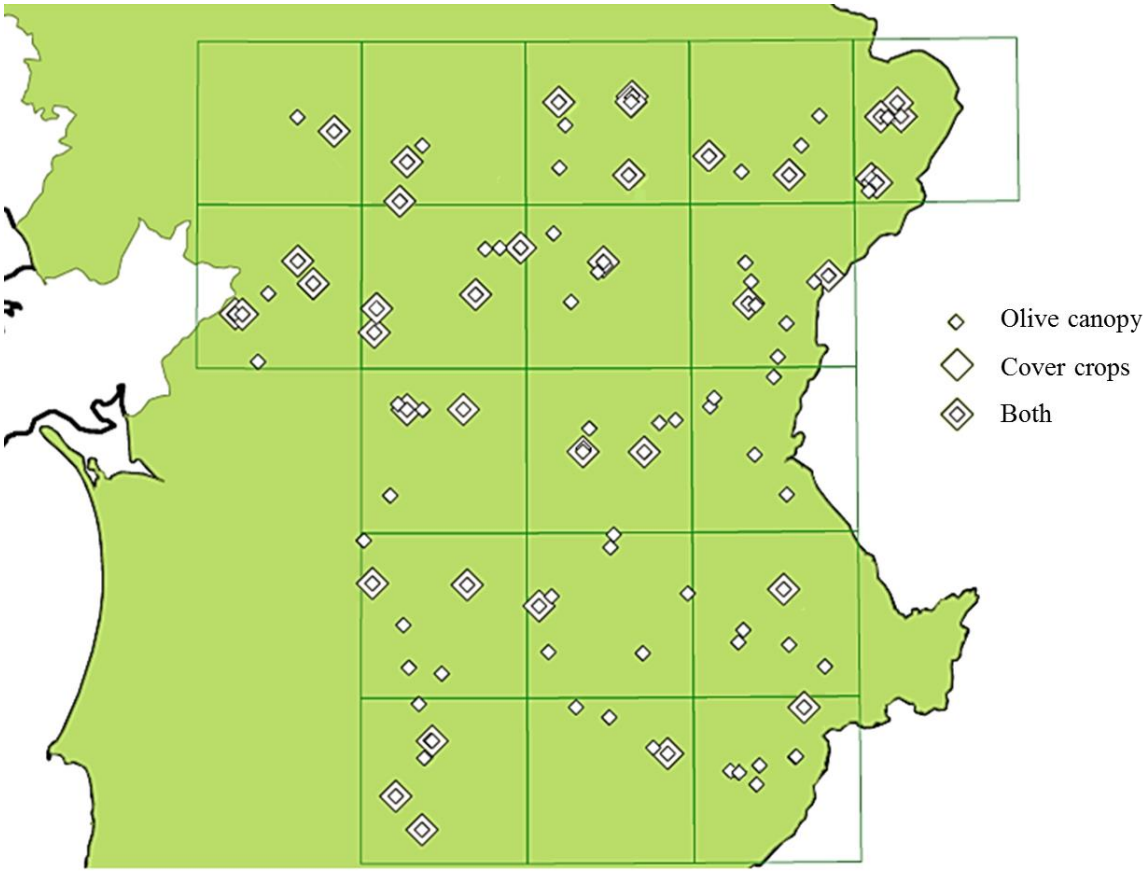

Figure 9: Map of the study area with the distribution of the sampling sites and the host plants sampled at each site.

4.2 Field collection of insects

For the purpose of this study 115 capture sites were selected. The sampling took place from October 25th to November 15th of 2016. Insects were collected using a suction technique with a modified vacuum device, a John W. Hock Company gasoline-powered Agricultural Backpack 2-Cycle Aspirator Model 1612 with a 12.7 cm diameter collection nozzle (126.68 cm^2) and a 64 km/h air intake. This method allows us to standardize sampling amongst different types of plants (i.e., herbaceous, shrubs, and trees). At each location, five randomly selected olive trees were vacuum-sampled around the canopy for ten

seconds each, and the collected arthropods pooled into a sampling unit (hereafter referred as local olive sample). When present, ground cover spontaneous plants were also sampled for fifty seconds, forming another sampling unit (hereafter referred as local ground cover sample).

Thereby, each sampling site has one or two sampling units, depending if cover crops are locally absent or present. Collected samples were preserved in a freezer at -20 ºC until laboratory sorting and identification.

4.3 Screening and morphological identification

Samples of insects were initially counted and sorted by taxonomic order following Chinery (1988) and were preserved in microtubes containing 70% ethanol, and parasitoids were further sorted and classified into morphospecies according to their similarities. Morphospecies did not involve the identification of species *per se*, but rather the separation of taxa based on morphological characters that were easily observable.

After, the individuals were identified under a stereoscopic microscope coupled with a camera to the lowest taxonomic levels like family or subfamily (when possible) following the key of identification proposed by Goulet, H. & Hubert, J. F. (1993) based on their morphological and physical characteristic. Once identified, each morphospecies were labelled accordingly to its capture site, stored in 70% ethanol and maintained at 4 ºC.

In order to allow further confirmation of identification, photographs were taken and to confirm morphological taxonomic identification and to contribute to the Barcode of Life, an attempt was made to amplify and sequence the COI amplicon of the sampled parasitoids.

All preserved specimens were deposited in the Entomology Laboratory at ICAAM/UÉvora, Évora, Portugal.

4.4 Species selection for molecular identification

The selection of target species for DNA barcoding was a non-random process; it was based on an informed combination of methodological requirements and research considerations. Target species were chosen based on their taxonomic groups, with priority being given to those groups whose potential has proven to be effective in controlling olive pests -essentially those parasitoids from chalcidoidea and ichneumonoidea superfamilies.

4.5 DNA extraction

Total genomic DNA was extracted from an individual insect from each of the selected morphospecies according to the manufacture's protocol for the NZY Tissue gDNA Isolation Kit (Lisbon, Portugal), with an overnight incubation step. The method is a spin column silica-based and requires no phenol or chloroform extraction. This kit uses optimized lysis buffers containing Proteinase K and SDS to release DNA from cells.

After preparing the lysate, DNA is selectively absorbed into the NZYSpin Tissue Column and other impurities such as proteins and salts are removed during the washing steps. The eluted genomic DNA had an A260/280 ratio between 1.7 and 1.9, suitable to use in downstream applications like PCR for sequencing.

4.6 Polymerase chain reaction (PCR) analysis

After DNA extraction, an amplicon of the mitochondrial gene of cytochrome oxidase subunit I (COI) was amplified by PCR using the universal invertebrate barcoding primers LCO1490 (5'- GGTCAACAAATCATAAAGATATTGG3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al*., 1994).

Each PCR reaction consisted of 0.25 µL of dNTPs, 0.125 µL of polymerase (i-taq, NZYTech – Genes & Enzymes), 0.25 µL of each primer, 2.5 µL of PCR buffer, 1.0 µL DNA extraction, and deionized water to bring the total reaction volume to 12.5 µL.

PCR temperature cycles were carried out in a GeneAmp® PCR System 2720 thermocycler (Applied Biosystems, USA) and the typical thermal cycling profile consisted of an initial denaturation step at 94 ºC for 4 minutes, followed by 35 cycles of 94 °C for 30 seconds, 53 °C for 30 seconds, and 72 °C for 1 minute. The last cycle was followed by 10 minutes at 72 °C to complete any partially synthesized strands.

Amplified products were stored at 4 ºC in the original PCR mix.

All PCR products were checked for bands and the separated genomic DNA was visualized using gel documentation. The isolated DNA was loaded on 1% agarose electrophoresis gel stained with 2 µL of GreenSafe Premiun (NZYTech – Genes & Enzymes, Portugal) and run for 90 min at 80 V. Molecular weight was identified with 1 Kb DNA ladder (NZYTech – Genes & Enzymes, Portugal), through UV transillumination.

In all reactions performed, there were included a negative control to assess the presence and / or absence of possible contaminants or inhibitors during the process of DNA extraction and / or preparation of PCR reactions.

4.7 Sequencing and Sequence analysis

The purification of the sequencing products obtained was carried out following the manufacture's protocol for the NZYGelpure kit (Lisbon, Portugal). The method is designed for direct purification of PCR products and utilizes a silica-gel based membrane which selectively adsorbs DNA fragments in the presence of specialized binding buffers, while other impurities that do not bind to the membrane and are washed away. DNA fragments are then eluted off the column and can be used for downstream protocols without further processing. After this procedure they and were stored at -20 ºC.

Sequencing reactions were carried out by a specialized company (Eurofins SA.).

The DNA sequences were aligned and analyzed using the software GeneStudio, Inc. (Suwanee, GA, USA). The DNA and deduced amino acid sequences were submitted to NCBI-BLAST (Basic Local Alignment Search Tool) from NCBI's GenBank for confirmation of the taxonomic positioning status defined a priori. After obtaining the

molecular results, the specimens were re-examined and their morphological identification reappraised.

4.8 Data analyses

General characterization of parasitoids

The numbers of individuals obtained in each sampling site during the collection period were grouped into morphospecies, superfamilies, families and subfamilies.

The overall abundance (total number of individuals obtained), richness (number of corresponding morphotypes) and relative frequency (number of individuals captured in relation to the total sample) of these species across the different strata were analyzed.

Relative frequency

Relative Frequency (Rf) was calculated according to the formula:

$$Rf = (n / N) * 100$$

Where n = number of individuals collected from each family; N = total number of individuals collected in the study.

Diversity index

Diversity of insect species was calculated using Shannon-Weaver diversity index (H) (Shannon & Weaver 1963). Shannon's index was selected as a measure of diversity as it is widely used in ecological studies and not very sensitive to rare species and sample size (Scalercio, *et al*., 2012).

The Shannon index is given by the formula below:

$$H = -\Sigma pi\ lnpi$$

Where pi = S/N, S is the total number of individuals of one species, N is the total number of all individuals in the sample and ln = logarithm to base e. The proportion of species relative to total number of species (pi) was calculated and multiplied by natural logarithm of this proportion (ln pi). The results were summed across the species and multiplied by -1.

Weather data

To determine the effects of climatic variables on parasitoids abundance the number of individuals captured was correlated with factors, such as:

a) Maximum and average summer temperatures, both obtained from mean values of the summer months, from July and August.
b) Total rainfall (in mm) recorded during the month of September and summer rainfall as average values of summer months, from July to August.
c) Total number of heatwave days and average of heatwave days, considering the sum of the total days of each heatwave period and number heatwaves recorded on summer months

Data were obtained directly from weather stations of the Instituto Português do Mar e da Atmosfera (IPMA), located near a sampling site or by inverse distance weighted (IDW) interpolation method, when sampling sites were apart from weather stations.

Weather observations were converted into group intervals in the overall data set and tables defining the rank values used for testing differences, between groups of each variable, are presented below (Tables 3 - 8).

Table 3: Average summer temperature and associated rank intervals for data analysis.

Rank	Temperature (°C)
1	< 23.5
2	23.6 - 24.0
3	24.1 - 24.5
4	> 24.6

Table 4: Maximum summer temperature and associated rank intervals for data analysis.

Rank	Temperature (°C)
1	< 31,5
2	31.6 - 32.0
3	32.1 - 32.5
4	32.6 - 33.0
5	33.1 - 33.5
6	>33,6

Table 5: Total amount rainfall during the month of September and associated rank intervals for data analysis.

Rank	Rainfall (mm)
1	< 9,5
2	9.6 - 11.5
3	11.6 - 13.5
4	13.6 - 15.5
5	15.6 - 17.5
6	> 17.6

Table 6: Average summer rainfall and associated rank intervals for data analysis.

Rank	Rainfall
1	< 4,5
2	4.6 - 5.0
3	5.1 - 5.5
4	5.6 - 6.0
5	6.1 - 6.5
6	6.6 - 7.0
7	7.1 - 7.5
8	7.6 - 8.0
9	> 8.0

Table 7: Total days of heatwaves and associated rank intervals for data analysis.

Rank	Days
1	< 95
2	96 - 100
3	101 - 105
4	106 - 110
5	111 - 115
6	> 116

Table 8: Average of days of heatwave and associated rank intervals for data analysis.

Rank	Days
1	< 32
2	33 - 34
3	35 - 36
4	37 - 38
5	> 39

Landscape complexity and dimension

In order to define the scale at which landscape variables exhibit stronger effects on populations' abundance, a landscape analysis was performed at different spatial extents.

Three circular areas, with radii of 0.25 km, 0.5 km and 1 km were nested around each sampling site. Data provided by QGIS software were used to assess the different types of land use and their proportion (area) within each circle to determine the specific landscape features.

The different land cover classes present across study sites were identified as olive groves, streams, pastures, vineyard and Montado habitat, comprising Cork and Holm oaks, and its area was converted into interval groups representing the percentage of occupancy. Rank transformed land cover data was later tested for differences within each buffer size considering each one of the particular type of land use (Table 9).

Table 9: Percentage of land cover area and associated rank intervals for data analysis

Rank	Area (%)
0	< 5%
1	6 - 10%
2	11 - 25%
3	26 - 50%
4	51 - 75%
5	> 76%

The proximity of neighboring olive groves and streams was calculated as the distance (km) "as the crow flies" from the site centroid to the closest point of the feature. Data was later converted to ranks of intervals of distance according to their proximity and groups were tested for differences (Table 10).

Table 10: Distance from olive groves and streams and associated rank intervals for data analysis.

Rank	Distance (m)
1	< 25
2	26 - 50
3	51 - 100
4	101 - 200
5	201 - 400
6	401 - 600
7	601 - 1000
8	> 1000

Statistical analysis

Statistical analyses were performed using the non-parametric Kruskal-Wallis test as the data assumptions for parametric statistics (normality and heteroscedasticity) are violated. This analysis allows the ranking of the dependent (abundance of the respective taxon) and independent variables (host type and ecological factors) based on their explanatory importance. The overall differences among groups were revealed after a previous data rank transformation, followed by post-hoc test LSD to explore differences in pair-wise comparison of groups, at a significance level of 0.05 (Marôco, 2007).

The null hypothesis of the Kruskal–Wallis test is that the mean ranks of the groups are the same, meaning that parasitoid abundance and diversity of a given taxa from sampling sites are the same across explanatory variables. On the contrary, if the null hypothesis is rejected it suggests statistical significance and determine whether the difference was likely to be biologically meaningful.

Initially, the effect of the type of host plant (olive tree canopy or cover crops) on the average abundance of parasitoids captured was tested for differences. Superfamilies, families, subfamilies and morphospecies comprising ten or more individuals were analyzed.

As a second step, all taxa identified were tested for differences under several explanatory variables in order to identify the factors driving their abundance and spatial distribution. For this set of analysis captures associated to the olive tree canopies were exclusively considered, due to the major representativeness of these samples across the grids in the study area.

We used sampling grids (30 m x 30 m) to evaluate whether there is a significant variation on abundance of parasitoids in the presence of a spatial extent; climatic conditions recorded in the previous summer; average and total number of days associated with heatwaves; distance (m) from the sampling site to neighboring olive groves and streams and the complexity and dimension of different land cover classes within a radius of 250 m (for all landscape classes) and 500 m and 1000 m for vineyards and streams.

Finally, diversity was calculated using Shannon-Weaver index for each sampling site. An average index value was obtained for each stratum, and later olive tree canopies and cover crops were tested for differences comparing their parasitoid diversity. Likewise, an average index value was obtained for each grid and tested for differences in their diversity per strata independently.

Data analysis was performed on the IBM-SPSS software, version 20.0.0 (SPSS Inc., IBM Company, 2010).

5. Results

5.1 Overall abundance and community composition

A total of 1353 specimens of Hymenoptera parasitoids distributed amongst 9 superfamiles, 22 families and represented by 263 morphospecies, were collected in several olive orchards of Alentejo.

Results show a numerical similarity in abundance of olive canopies and the cover crops; however, these communities vary in their species composition (Table 11).

The great majority of the species belonged to 3 superfamilies. Altogether, Ichneumonoidea, Chalcidoidea and Platygastroidea comprised the major proportion of the total abundance found (1206 individuals, 89.14%). The remaining 6 superfamilies had very low abundance. In fact, all except Cynipoidea, Ceraphronoidea and Poroctotrupoidea were represented by less than 10 indviduals (Table 11).

However, the abundance of some taxa was relatively high, such as the families Braconidae (33.26%), Scelionidae (15.52%), Pteromalidae (11.01%), Eulophidae (8.80%) and Encyrtidae (5.62%), which were the most representative. The remaining families were less well represented and showed relative frequencies below 5% and the families Megalodontidae, Tetracampidae and Tiphiidae each had only one individual collected (Table 11).

Table 11: Checklist of taxon and abundance of insects recorded from olive trees and cover crops.

Insect taxon	Community		Total	RF(%)
Superfamily/ Family	Olive trees	Cover crops		
CERAPHRONOIDEA				
Ceraphronidae	17	12	29	2.14
Megaspilidae	7	10	17	1.26
CHALCIDOIDEA				
Aphelinidae	1	62	63	4.66
Encyrtidae	44	32	76	5.62
Eulophidae	19	100	119	8.80
Eupelmidae	1	9	10	0.74
Mymaridae	8	33	41	3.03
Perilampidae	1	1	2	0.15
Pteromalidae	75	74	149	11.01
Tetracampidae	1	0	1	0.07
Thrichogrammatidae	0	6	6	0.44
CHRYSIDOIDEA				
Bethylidae	4	2	6	0.44
CYNIPOIDEA				
Figitidae	8	49	57	4.21
ICHNEUMONOIDEA				
Braconidae	113	337	450	33.26
Ichneumonidae	22	24	46	3.40
MEGALODONTOIDEA				
Megalodontidae	1	0	1	0.07
PLATYGASTROIDEA				
Platygastridae	14	19	33	2.44
Scelionidae	159	51	210	15.52
PROCTOTRUPOIDEA				
Diapriidae	11	8	19	1.40
Proctotrupidae	2	0	2	0.15
VESPOIDEA				
Tiphiidae	1	0	1	0.07
Vespidae	2	1	3	0.22
N/D	6	6	12	0.89
Total number of insect individuals	517	836	1353	100.00
Total number of insect families	21	18		

RF = Relative frequency of hymenoptera parasitoid families in relation to the total of parasitoid hymenoptera collected.

Additionally, at subfamily level, individuals were substantially more abundant in Braconidae family (12 subfamilies, 450 individuals) than in Ichneumonidae family (11 subfamilies, 46 individuals).

Members of Braconidae family were mostly distributed in Alysiinae, Gnamptodontinae and Opiinae subfamilies, together they accounted for 88.30% of the total captured braconids. While the Ichneumonidae was mostly represented by Phygadeuontinae, Cryptinae and Ichneumoninae accounting for 72.73% of the total ichneumonids captured.

Although some parasitoids from both communities could not be identified due to their poor condition, they represented less than 1% of the samples collected.

5.2 Effect of host on parasitoid abundance and diversity

In this study we investigated different explanatory variables related to parasitoid abundance and diversity on plants. We found that the type of host (olive canopy or cover crop) can significantly ($p < 0.05$) affect parasitoid communities, but that their effects differ among diferent taxonomic groups. Cover crops were associated with a higher number of morphospecies belonging mainly to Aphelinidae (Figures 9-11), Encyrtidae (Figure 20), Eulophidae (Figures 21 and 22), Pteromalidae (Figures 25-27), Figitidae (Figures 23 and 24) and Braconidae (Figures 12-19) families. As opposed, in the canopies of the olive trees, higher number of individuals captured was was associated only with the Selionidae (Figures 28 - 30) family (Table 12).

Photographic images of representative morphospecies are presented in the annex.

Table 12: Total abundance, mean ± standard error and significance of morphospecies captured in olive canopies and cover crops.

Family/Specie	Olive canopy				Cover crops				Sig.
	Total	Mean		SE	Total	Mean		SE	
APHELINIDAE									
Aphelinidae sp1	0	0.00	±	0.000	10	0.27	±	0.148	*
Aphelinidae sp2	0	0.00	±	0.000	25	0.68	±	0.676	
Aphelinidae sp3	0	0.00	±	0.000	10	0.27	±	0.270	
BRACONIDAE									
Asobara sp.	0	0.00	±	0.000	11	0.30	±	0.173	*
Braconidae sp1	0	0.00	±	0.000	85	2.30	±	0.625	***
Braconidae sp2	0	0.00	±	0.000	10	0.27	±	0.158	*
Chorebus sp.	0	0.00	±	0.000	23	0.62	±	0.278	**
Dinotrema sp1	16	0.43	±	0.132	36	0.97	±	0.394	
Dinotrema sp2	4	0.11	±	0.065	38	1.03	±	0.350	*
Dinotrema sp3	0	0.00	±	0.000	16	0.43	±	0.253	*
Opius sp.	12	0.32	±	0.155	27	0.78	±	0.315	
ENCYRTIDAE									
Encyrtidae sp1	0	0.00	±	0.000	21	0.57	±	0.407	*
EULOPHIDAE									
Euderus albitarsis	1	0.03	±	0.027	43	1.16	±	0.579	***
Euplectrus flavipes	0	0.00	±	0.000	10	0.27	±	0.167	*
FIGITIDAE									
Figitidae sp1	0	0.00	±	0.000	18	0.49	±	0.163	**
Figitidae sp2	0	0.00	±	0.000	10	0.27	±	0.074	**
PTEROMALIDAE									
Pteromalidae sp1	0	0.00	±	0.000	14	0.38	±	0.161	*
Pteromalidae sp2	0	0.00	±	0.000	17	0.46	±	0.153	**
Pteromalidae sp3	3	0.08	±	0.060	16	0.43	±	0.188	*
SCELIONDAE									
Telenomus sp1	11	0.30	±	0.109	23	0.62	±	0.210	
Telenomus sp2	16	0.43	±	0.231	1	0.03	±	0.027	*
Telenomus sp3	12	0.32	±	0.186	0	0.00	±	0.000	*

* -Sig <0.05
** - Sig 0.001<> 0.002
*** - Sig 0.000

In addition, in terms of diversity, statistical evaluation of means per community was analyzed and indicated a significant difference between the hosts. The Shannon-Weiner diversity index was compared by the Kruskal-Wallis test and reported the highest average value for the cover crops (Cover crops: *H'* = 0.0826 ± 0.0095; Olive canopy: H' = 0.0410 ± 0.0050, $p < 0.002$) (Mean ± SE).

A comparison of insect number between olive canopies and cover crops within superfamilies indicated that the two communities differed significantly ($p < 0.001$). It is noteworthy that the number of parasitoids collected under the superfamilies Chalcidoidea, Cynipoidea and Ichneumonoidea were consistently higher in the cover crops than in the canopies (Table 13).

Table 13: Total abundance, mean ± standard error and significance of superfamilies captured in olive canopies and cover crops.

Superfamily	Olive tree				Cover crop				Sig.
	Total	Mean		SE	Total	Mean		SE	
Ceraphronoidea	5	0.135	±	0.057	22	0.595	±	0.137	**
Chalcidoidea	61	1.649	±	0.329	296	8.000	±	1.462	***
Chrysidoidea	2	0.054	±	0.038	2	0.054	±	0.038	
Cynipoidea	4	0.108	±	0.052	46	1.243	±	0.299	***
Ichneumonoidea	51	1.378	±	0.311	330	8.919	±	1.620	***
Megalodontoidea	1	0.027	±	0.027	0	0.000	±	0.000	
Platygastroidea	53	1.432	±	0.432	65	1.757	±	0.345	
Proctotrupoidea	5	0.135	±	0.057	7	0.189	±	0.076	
Vespoidea	0	0.000	±	0.000	1	0.027	±	0.027	

* -Sig <0.05
** - Sig 0.001<> 0.002
*** - Sig 0.000

Grouped by families, the number of parasitoids collected was found to be significantly different ($p < 0.001$), since a major proportion of individuals of Aphelinidae, Braconidae, Eulophidae, and Mymaridae families were found in the cover crops. Although other families, namely Ceraphronidae, Eupelmidae, Megaspilidae, Platygastridae and Pteromalidae presented a less strong significance ($p < 0.005$) they still differ from the olive canopies. The remaining families, with few or no individuals did not show any difference between the strata compared (Table 14).

Table 14: Total abundance, mean ± standard error and significance of families captured in olive canopies and cover crops.

Family	Olive tree				Cover crop				Sig.
	Total	Mean		SE	Total	Mean		SE	
Aphelinidae	0	0.00	±	0.000	57	1.54	±	0.741	***
Bethylidae	2	0.05	±	0.038	2	0.05	±	0.038	
Braconidae	37	1.00	±	0.209	310	8.38	±	1.598	***
Ceraphronidae	2	0.05	±	0.038	12	0.32	±	0.117	*
Diapriidae	4	0.11	±	0.052	7	0.19	±	0.076	
Encyrtidae	19	0.51	±	0.143	31	0.84	±	0.436	
Eulophidae	8	0.22	±	0.079	90	2.43	±	0.699	***
Eupelmidae	0	0.00	±	0.000	8	0.22	±	0.088	*
Figitidae	4	0.11	±	0.052	46	1.24	±	0.299	***
Ichneumonidae	14	0.38	±	0.147	20	0.54	±	0.148	
Megalodontidae	1	0.03	±	0.027	0	0.00	±	0.000	
Megaspilidae	3	0.08	±	0.045	10	0.27	±	0.074	*
Mymaridae	3	0.08	±	0.045	32	0.86	±	0.258	***
Perilampidae	0	0.00	±	0.000	1	0.03	±	0.027	
Platygastridae	5	0.14	±	0.057	17	0.46	±	0.120	*
Proctotrupidae	1	0.03	±	0.027	0	0.00	±	0.000	
Pteromalidae	30	0.81	±	0.225	71	1.92	±	0.416	*
Scelionidae	48	1.30	±	0.410	48	1.30	±	0.322	
Tetracampidae	1	0.03	±	0.027	0	0.00	±	0.000	
Trichogrammatidae	0	0.00	±	0.000	6	0.16	±	0.091	
Vespidae	0	0.00	±	0.000	1	0.03	±	0.027	

* -Sig <0.05
** - Sig 0.001<> 0.002
*** - Sig 0.000

Composition of subfamily assemblage likely showed a significant output when Kruskal-Wallis test was applied. Significant differences in the abundance (p = 0.002) were found for the Alysiinae (Cover crops: 3.432 ± 0.856; Olive canopies 0.541 ± 0.143) (mean ± SD), being the most dominant subfamily present in both strata (Table 15).

The presence of subfamily Gnamptodontinae was notorious in cover crops (2.919 ± 0.848, p = 0.000), followed Aphidiinae (0.432 ± 0.200, p = 0.005) which were subfamilies exclusive from this stratum (Table 15).

Table 15: Total abundance, mean ± standard error and significance of subfamilies captured in olive canopies and cover crops.

Subfamily	Olive tree				Cover crop				Sig.
	Total	Mean		SE	Total	Mean		SE	
Alysiinae	20	0.541	±	0.143	127	3.432	±	0.856	**
Aphidiinae	0	0.000	±	0.000	16	0.432	±	0.200	*
Braconinae	0	0.000	±	0.000	2	0.054	±	0.054	
Cryptinae	0	0.000	±	0.000	4	0.108	±	0.065	
Diplazontinae	0	0.000	±	0.000	1	0.027	±	0.027	
Doryctinae	0	0.000	±	0.000	8	0.216	±	0.079	
Eucerotinae	1	0.027	±	0.027	1	0.027	±	0.027	
Euphorinae	1	0.027	±	0.027	9	0.243	±	0.147	
Gnamptodontinae	0	0.000	±	0.000	108	2.919	±	0.848	***
Ichneumoninae	1	0.027	±	0.027	3	0.081	±	0.045	
Mesochorinae	0	0.000	±	0.000	1	0.027	±	0.027	
Meteorinae	1	0.027	±	0.027	0	0.000	±	0.000	
Microgastrinae	3	0.081	±	0.045	0	0.000	±	0.000	
Opiinae	12	0.324	±	0.155	37	1.000	±	0.376	
Orgilinae	0	0.000	±	0.000	1	0.027	±	0.027	
Orthocentrinae	0	0.000	±	0.000	1	0.027	±	0.027	
Phygadeuontinae	12	0.324	±	0.145	5	0.135	±	0.057	
Rogadinae	0	0.000	±	0.000	5	0.135	±	0.088	
Sigalphiinae	0	0.000	±	0.000	1	0.027	±	0.027	

* -Sig <0.05
** - Sig 0.001< > 0.002
*** - Sig 0.000

5.3 Effects of spatial scale on parasitoids abundance and diversity

The spatial effect on parasitoid abundance and diversity was analyzed within each grid scale, considering the totality of 27 grids (30 x 30 km) that comprised the whole sampling area. The average Shannon index obtained per square grid considering each strata independently did not differ significantly (Cover crops: H' = 0.0814 ± 0.0086; Olive canopy: H' = 0.0379 ± 0.0031), showing no spatial influence of this variable on parasitoid diversity.

Considering the olive canopy stratum, the spatial effect on parasitoid abundance showed significant differences ($p < 0.05$). Variations in spatial distribution of parasitoids at some taxonomic levels were restricted to only 3 grids (0, 26 and 27).

Results revealed that grid 0 held significantly more individuals captured belonging to the Platygastroidea superfamily (16.33 ± 10.171, $p = 0.026$); differences were also detected reaching lower levels from the same taxonomic group. Captures were significantly higher for Scelionidae family (16.00 ± 10.214, $p = 0.012$) in contrast to the other families and for one morphospecies of the genus *Telenomus sp.* (0.18 ± 0.071, $p = 0.026$). As regards to grids 26 (0.60 ± 0.400, $p = 0.049$) and 27 (0.57 ± 0.297, $p = 0.05$) they differed from the rest presenting significantly higher catches of Platygastroidea and Proctotrupoidea superfamily respectively.

5.4 Effect of ecological variables on parasitoids abundance

We hypothesized that populations of natural enemies within the olive grove may be affected by adjacent natural landscape, and their response might differ depending on their presence, dimension and proximity.

Results showed that landscape features did not influence the overall abundance; however, they did affect some parasitoid families individually ($p < 0.05$). Significant differences were detected when 11 to 25% of landscape occupied by streams areas was reported within a 1000 m² radius, recording more hymenopteran parasitoids of Ceraphronidae family (0.15 ± 0.045, $p = 0.026$). Conversely, Diapriidae (0.10 ± 0.034, $p = 0.008$), Megaspilidae (0.06 ± 0.023, $p = 0.003$), Mymaridae (0.07 ± 0.028, $p = 0.004$) and Platygastridae (0.13 ± 0.034, $p = 0.018$) abundance was significantly increased when the areas of Holm oak corresponded to 26 to 50%, but only within a 250 m² radius. Captures of parasitoids belonging to families Megaspilidae (0.06 ± 0.023, $p = 0.004$) and Scelionidae (1.42 ± 0.363, $p = 0.040$) were significantly higher when 10 to 25% of Cork oak areas within a 250 m² radius encompassed the sampling sites.

Regarding taxonomic morphospecies, landscape within a 250 m² radius indicated an increment of parasitoid population size. Higher numbers of specimens of the genus *Dinotrema sp.* (0.18 ± 0.129, p = 0.024) were noticed when 2 to 25% of landscape were occupied by vineyard areas adjacent to capture sites. Additionally, landscape composed by 5 to 25% of cork oak significantly differed from the others, showing more captures of the species *Euderus albitarsis* (0.03 ± 0.015, p < 0.05) and *Telenomus sp.* (0.18 ± 0.071, p = 0.00) respectively.

No influence of surrounding landscape was recorded at superfamily or subfamily levels and no taxa were related to significant captures at longer distances.

A few significant interactions occurred between parasitoids and environmental variables namely temperature and rainfall. There was a strong relation of temperature registered in the previous summer affecting parasitoid population, once significant differences were observed when maximum temperature do not exceede 31.5 °C in summer season providing greater captures of genus *Telenomus sp.* (0.24 ± 0.76, p = 0.03), whereas captures of the genus D*inotrema sp.* was recovered in great number (0.40 ± 0.075, p = 0.005) when temperatures reached a maximum of 32 °C. Likewise, significantly more captures of superfamily Proctotrupoidea (0.10 ± 0.034, p = 0.011) was recorded when the average summer temperatures ranged from 23.6 ºC to 24 ºC.

No significant differences were observed regarding the total days of heatwaves that occurred in the summer of 2016 except for Diapridae captures (0.10 ± 0.034, p = 0.008), significantly higher when an interval of 96 to 100 total days of heat waves were recorded during the summer, although the average of 37 to 38 days of heat waves did differ (0.40 ± 0.075, p = 0.004) for the braconid *Dinotrema sp*. corresponding to the period with more captures.

The effect of rainfall was observed affecting some taxa. Highest captures of superfamily Cynipoidea were recorded when the total amount of rainfall recovered during the month of September did not exceed a total value of 11.5 mm (0.22 ± 0.147, p = 0.008). Besides, captures of family Figitidae (0.07 ± 0.025, p = 0.008) were significantly higher when rainfall registered even lower values, below 9.5 mm, during September as well, the month prior the captures.

At the subfamily level, no temperature and rainfall influenced insect population.

5.5 DNA barcoding of selected morphospecies

We aimed to cover a range of the criteria previously mentioned, since the number of species across families is limited, preference was given to morphospecies belonging mainly to chalcidoidea and ichneumonoidea superfamilies. Universal invertebrate barcoding primers LCO1490 and HCO2198 used are very robust, allowing that the majority of COI amplifications occurred without major problems. Nevertheless, it failed to amplify accurately this specific segment for some families such as Encyrtidae, Aphelinidae, Figitidae and Megaspilidae. Several PCR reactions were tested, using different concentrations of reagents and different PCR thermal cycles, without any successful result.

One representative of each morphospecies selected was sequenced and a total of 27 sequences representing the most abundant families were obtained. The alignment was straightforward, and no insertions or deletions have been found. Well defined peaks in chromatograms and the absence of stop codons indicated that amplification of pseudogenes did not occur. The results showed that the amplified PCR products resulted in sequences varying from 643 to 658 bp in length. The target fragment incorporates the DNA barcode region of the animal taxa and sequence diversity in this region was used as a tool for species discrimination (Figure 10). The online database used allowed species identification when our sequence matched the available reference sequence with an identity value greater than 97%, given that intraspecific genetic distance should not exceed 3% (Hebert *et al*., 2003a) (Table 16).

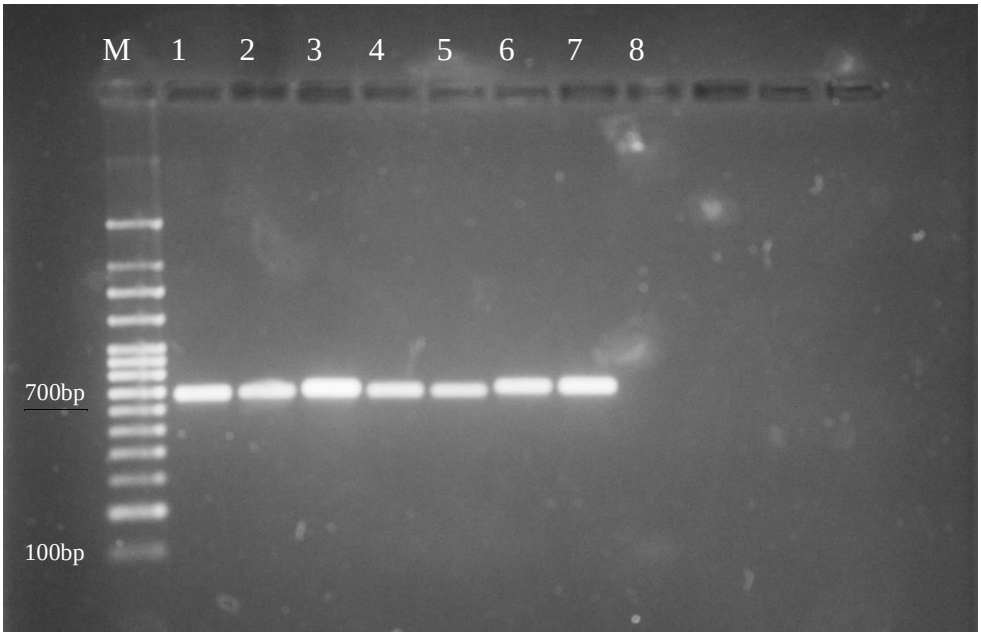

Figure 10: Example of an amplified COI fragments on agarose gel electrophoresis of PCR product. PCR product was performed using 1% (w/v) agarose gel with 2 µL of GreenSafe Premiun. Each well has each sample DNA loaded. Well No. M ladder 100-1000bp, Well No.1 - 7 genomic DNA, Well No. 8 Negative Test Control.

Table 16: Representative specimens collected in sampling sites of olive trees and cover crops with GenBank accession numbers of COI.

ID	Assigned barcoding species				Similarity with online data base		Sequence acession number
	Superfamily	**Family**	**Subfamily**	**Genus/ Species**	**Base pair**	**Percentage**	
2	Chalcidoidea	Eulophidae	Euderinae	*Euderus albitarsis*	482/537	90%	MG836467.1
4	Platygastroidea	Scelionidae	Telenominae	*Telenomus*	557/613	91%	KT896659.1
16	Platygastroidea	Scelionidae	Telenominae	*Telenomus*	557/611	91%	KR879424.1
31	Platygastroidea	Platygastridae	Platygastrinae	*Leptacis*	600/616	97%	KR800540.1
36	Platygastroidea	Scelionidae	Telenominae	*Telenomus*	554/607	91%	MG353138.1
40	Chalcidoidea	Eulophidae	Eulophinae	*Euplectrus flavipes*	566/606	93%	MH587859.1
43	Proctotrupoidea	Diapriidae	Diapriinae	*Trichopria*	573/587	98%	JF863269.1
63	Chalcidoidea	Pteromalidae	Pteromalinae	*Cecidostiba fungosa*	538/599	90%	JQ417026.1
95	Chrysidoidea	Bethylidae	Epyrinae	*Rhabdepyris*	533/615	87%	HQ930224.1
114	Ichneumonoidea	Braconidae	Alysiinae	*Phaenocarpa*	529/535	99%	MG442957.1
122	Ichneumonoidea	Braconidae	Opiinae	*Opius*	580/621	93%	MF932497.1
124	Ichneumonoidea	Braconidae	Alysiinae	*Dinotrema*	614/630	97%	KR803141.1
129	Ichneumonoidea	Braconidae	Euphorinae	*Microctonus hyperodae*	487/566	86%	EU078360.1
133	Ichneumonoidea	Braconidae	Microgastrinae	*Apanteles biplagae*	585/614	95%	MH059600.1
134	Ichneumonoidea	Braconidae	Alysiinae	*Dinotrema*	603/623	97%	MN671135.1
154	Platygastroidea	Scelionidae	Telenominae	*Telenomus*	488/541	90%	KM995940.1
157	Chalcidoidea	Eulophidae	Euderinae	*Euderus albitarsis*	530/603	88%	MG836469.1
207	Ichneumonoidea	Ichneumonidae	Phygadeuontinae	*Palpostilpnus*	520/613	85%	KY998804.1
239	Ichneumonoidea	Braconidae	Alysiinae	*Dacnusa*	522/616	85%	KR899807.1
242	Ichneumonoidea	Braconidae	Alysiinae	*Chorebus*	528/552	96%	MK532727.1
243	Ichneumonoidea	Braconidae	Aphidiinae	*Lysiphlebus fabarum*	574/574	100%	HQ724552.1
245	Ichneumonoidea	Braconidae	Alysiinae	*Exotela*	523/566	92%	AY935346.1
246	Ichneumonoidea	Braconidae	Alysiinae	*Dinotrema*	628/637	98%	JX832106.1
250	Ichneumonoidea	Braconidae	Alysiinae	*Asobara*	564/564	100%	KR783522.1
251	Ichneumonoidea	Ichneumonidae	Cryptinae	*Gelis*	433/507	85%	KR885096.1
259	Platygastroidea	Platygastridae	Platygastrinae	*Leptacis*	392/460	85%	MG502769.1
262	Ichneumonoidea	Braconidae	Meteorinae	*Meteorus pendulus*	408/412	99%	HQ263992.1

Nevertheless, a few morphological and molecular identifications were not congruent. For example, morphospecies 31, morphologically identified as Scelionidae, matched with a reference sequence identified as Platygastridae at family level with an identity value of 97%. Another case occurred with morphospecies 262 initially identified as Doryctinae matches with a reference sequence identified as Meteorinae at subfamily level and had over 99 % similarity with those sequences annotated in GenBank.

Using molecular tools, only 8 morphospecies were successfully fully identified at species level, where, in most of the cases, identification was verified only at genus level. For instance, all morphospecies, with exception of morphospecies 31 matched morphological characters and molecular information to family level.

In addition, when COI sequences were analyzed in GStudio, it was verified that morphospecies 4 and 16 obtained sequences virtually identical, leading to the same result of molecular identification, and leading to the assumption that they belong to the same species and that the morphological differences were intra-specific. Likewise, morphospecies 2 and 157 belongs to the same species, *Euderus albitarsis*.

The subfamilies Alysiinae and Telenominae were ranked as the first and second most abundant subfamilies analyzed by molecular data and are mostly represented by the genus *Dinotrema* sp. and *Telenomus sp.* respectively.

6. Discussion

Identification of sampled insect parasitoids and their putative relevance in the ecossystem

The results of this study show a varied complex of parasitoids that naturally occur in the olive orchards of Alentejo: a total of 1353 parasitoids were sampled, belonging to 22 families. The families most captured with respect to the total number of individuals from the two strata (olive trees and cover crops) were as follows: Braconidae (450), Scelionidae (210), Pteromalidae (149), Eulophidae (119) and Encyrtidae (76). Those families have been reported in the literature as typically abundant groups in olive groves (Ruano *et al*., 2000), and some play an important role in the biological control of olive pests (Morris *et al*., 1999).

Previous studies have reported similar results for the composition of Hymenoptera captured in olive groves in several Mediterranean countries. Viggiani *et al*. (1997) reported that captures of Hymenoptera were dominated by families such as Aphelinidae, Braconidae, Ceraphronidae, Encyrtidae, Eulophidae, Ichneumonidae, Mymaridae, Platygastridae,

Scelionidae and Trichogrammatidae; while Herz *et al*., 2005 emphasized the frequency of parasitoids observed in olive groves, especially those belonging to the Braconidae, Eulophidae, Ichneumonidae, and Trichogrammatidae families. In studies conducted by Torres & Bueno (2000), the Ichneumonidae and Braconidae family were present in their captures in almost all olive groves and times of the year, but especially during the spring. Also according to the same authors, among the Chalcidoidea sampled, it was possible to identify the families Pteromalidae and Trichogrammatidae.

Furthermore, Teixeira *et al*. (2000) carried out a survey regarding the auxiliary fauna in olive groves managed under no phytosanitary treatments and observed that among the captured hymenopterans, the individuals that appeared most frequently belonged to the family Pteromalidae, Braconidae, Scelionidae, Eulophidae and Ichneumonidae. Also, hymenopteran collected from the ground herbaceous plants of olive orchards described by Villa *et al*. (2012) recorded that the most representative families were the Braconidae, Ichneumonidae, Scelionidae and Eulophidae; whereas Rodríguez *et al*. (2012) analysing the abundance of parasitoids in the olive canopy recorded that the most representative families were the Scelionidae, Pteromalidae and Encyrtidae, the latter accounted for more than 45% of all hymenoptera collected, an apparent discrepancy when compared to our results, which reported 5.62% of individuals collected of this family.

Analyzing the parasitoid fauna in olive groves in Portugal, Rei (2006) and Nave *et al*. (2017) observed that captures of Chalcidoidea were about twice as high as those of Ichneumonoidea; whereas in Spain, Ruano *et al*. (2000) registered that parasitoid Hymenoptera belonged mostly to the superfamily Chalcidoidea, corresponding to approximately to 90% of the captured specimens. The superfamily Ichneumonidae had a notorious presence in Greek olive groves, such as Broumas *et al*., (1973) and Neuenschwander (1982) observed, although in this study the captured Ichneumonoidea and Chalcidoidea were equally distributed in our samples, representing 37% and 35% of the total catches respectively.

Variations among results might arise from variables operating at a regional scale such as climatic conditions of the region at the time when the study was conducted, but also land use history or even surrounding natural or semi-natural vegetation (Landis *et al*., 2000).

A considerable variety of sampling methods for collecting individuals (i.e. sweep netting, beating, pitfall trapping, vacuum samples and yellow traps) may also influence the subset of species captured (Frazer *et al*., 2008). For that reason, comparisons are occasionally difficult due to differences in sampling effort and methodologies. Also, the deployment of traps in space and season usually produce highly heterogeneous results and seasonal pattern may mask diversity amongst species that cannot be reflected from short-term or "spot" samples, and that hinder comparative studies (New, 2012)

The great representativeness registered for the Braconidae and Scelionidae recorded in this study resembles the results obtained by other authors. Gonçalves (2016), in a survey carried out to contribute to the knowledge of the abundance and diversity of arthropods associated with the olive ecosystem under Integrated Pest Management highlighted that within the order hymenoptera, the predominance of Braconidae over other families was notorious and accounted for almost 34% of the overall abundance, a result close to our findings, in which the braconidae family accounted for 33.26% of the overall abundance.

As for the Scelionidae family, their significative presence in the captures was also observed by Rodríguez *et al*. (2012) and Paredes *et al*. (2013a). In their studies this family was among the most representative in the olive groves sampled, corresponding to about 16.3% of the overall abundance (Álvarez *et al*., 2019), a value similar to the one obtained in this study, in which the Scelionidae family accounted for 15.52% of the overall abundance.

Scelionids are endoparasitoids of insect eggs of most major orders and may be the reason why they are found in such great number, but also due to the fact of their abundande is related to warmer temperatures, as suggested our results, according to the notorious high temperatures occurred previously to the sampling period. Individuals identified in this study belonging to the genus *Telenomus* sp. exhibits a considerable parasitism capacity and longevity even at extreme temperatures (above 30 °C), indicating that can be well adapted to environments with such thermal conditions (Bruce *et al*., 2009), possibly under conditions associated with the effect of global warming, foreseen for the Iberian Peninsula. Although the abundance of this family was recorded in high numbers in our study, they remain not referred as natural enemies of the main olive pests (Teixeira *et al*., 2000) nor are they expected to be, facing their host preference. Members of the Braconidae family are all referred as parasitoids of cyclorrhaphous Diptera (Wharton, 1993). In habitat preferences,

too, most groups of braconids are characteristic of relatively warm and dry habitats (Mills, 1992).

As we studied the parasitoid complex present in olive orchards, we addressed the question whether the presence of these families of parasitoids may be associated with groups of insects that could be assumed as their potential hosts. Seven of the total families recorded in our study include species previously referred as important for the natural limitation of the main olive pests; namely, and in decreasing order of abundance, members of the families Braconidae, Pteromalidae, Eulophidae, Encyrtidae, Aphelinidae, Ichneumonidae and Eupelmidae, the latter being less represented (Teixeira *et al*., 2000; Torres, 2007). They belong mainly to two superfamilies, Chalcidoidea and Ichneumonoidea, which are of great importance due to the vast number of parasitoid species it includes and the large number of insect pests that are parasitized by members of these groups (Torres *et al*., 2007).

The diversity of the parasitoids observed in the olive groves reveals their importance as control agents and the accurate taxonomic identification of species has long been recognized as an essential first step in developing successful biological control programs (Hoddle *et al*., 2015).

The molecular analysis resulted in the identification of 19 parasitoids to genus level and 8 parasitoids to species level, although some amplification failures were recorded, suggesting less suitability of the universal primers used for these taxa (especially those belonging to Encyrtidae and Aphelinidae family). Using the same methodology, low amplification success rates have been reported for certain taxonomic groups, including nematodes (Derycke *et al*., 2010), Diptera (Van Houdt *et al*., 2010), marine invertebrates and, as we also report, for many species of hymenoptera (Yu *et al*., 2012). In fact, the failure to successfully amplify is not unusual for taxonomically DNA barcoding projects (Hajibabaei *et al*., 2006). Aside from presumably unsuccessful primer binding, which is probably the primary reason, failure to successfully recover DNA barcodes using universal primers, and depending on the target organism, may be due to failed sequencing reactions due to cross-contamination from other individuals in the mixture or the presence of competing COI sequence information (e.g., heteroplasmy and endosymbiotic bacteria) within individuals, or even degradation of DNA during collection or storage (Gibson *et al*., 2014). Yet, critical for DNA barcoding identification is either the availability of such

libraries of referenced DNA barcodes and the degree of taxonomic coverage of these libraries. So far, most taxa reference libraries are still largely incomplete (Virgílio *et al*., 2010), explaining the large number of morphospecies that remained to be identified in our study.

From the parasitoids identified to especies level, notably, one appears to have a dominant role in the control of *P. oleae*: *Euderus albitarsis* (Zetterstedt, 1838) has been referenced as belonging to the parasitoid complex of the genus *Prays* in the Mediterranean region (Moreno *et al*., 1990) and was reported for the first time in Portugal by Nave *et al*. (2017) in *P. oleae* anthophagous generation.

The species *Lysiphlebus fabarum* (Marshall, 1896), *Cecidostiba fungosa* (Geoffroy, 1785) and *Meteorus pendulus* (Müller, 1776) are referenced mainly as parasitoids of Diptera, Coleoptera, Lepidoptera and aphids (Stigenberg, *et al*., 2015; Gates *et al*., 2012; Kaldeh *et al*., 2012). Other taxonomic groups identified to genus level, members of the families, Scelionidae, Platygastridae, Diapriidae, Bethylidae, Ichneumonidae and especially Braconidae, the most important family containing tephritid parasitoids (Daane *et al*., 2011), and the apparent dominance of braconids over chalcidoid and other parasitoids should be further investigated to elucidate their suitability as new species as candidate agents for biological control of olive pests.

Influence of cover crops and canopy on parasitoids population abundance and diversity

The results of this study indicate that in general, ground cover vegetation had a positive effect upon parasitoids community, increasing their abundance and thus conferring the highest value of abundance and Shannon-Weiner index of parasitoids diversity when compared with the olive trees. Our data set was dominated by few taxa with high numbers and a large number of taxa represented by one or two individuals, which justifies the use of Shannon-Weiner index as a standard formula for calculating biodiversity, as it gives as much weight to those species which have few individuals as to those which have many individuals. On the contrary, with respect to Simpson index it gives more weight to common

or dominant species. In this case, species with only a few representatives will not affect the diversity, therefore in the present study the data produced based on Simpson index revealed a meaningless result and thus was not reported.

The presence of natural enemies in olive orchards and their relationship with olive trees and ground cover vegetation, likely correlates with habitat complexity, and according to Goméz *et al*. (2017) their abundance is increased when habitats have high numbers of plant species. It is known that highly structured and heterogeneous vegetation, as found in the most diverse cover crops in contrast to the simple stands found in the canopies, provides various resources food and sites for arthropods reproduction, colonization, and overwintering (Sobek *et al*., 2009), which can be expected to support a more abundant parasitoid community, and in some cases an increase of parasitism rates (Villa *et al*., 2016). Although we cannot make any assumptions about parasitism rates in our study, probably the higher parasitoid diversity in cover crops is due to more diverse vegetation, resulting in an increase of resources that natural enemies can exploit (Rusch *et al*., 2010).

We also expected that cover crops could influence the parasitoid composition present in the canopies, increasing morphospecies abundancy and diversity within the orchard ecosystem. However, few studies provide evidence of ground cover vegetation derived benefits upon the establishment of natural enemies of insect pests within the canopy of orchard trees; whereas some studies showed that cover crops favored beneficial arthropods in tree canopy as observed in olive orchards by Rodriguéz (2012) and Paredes *et al*. (2013a), others found that ground cover had little effect upon the density, or type, of arthropods reported in tree canopy by Bone *et al*., (2009) in apple orchards, Smith *et al*. (1996) in pecan orchards and Danne *et al*. (2010) in vineyards.

Landscape-scale factors related to the composition and proximity of vegetation including other crops, natural or semi-natural vegetation are known to affect the presence of natural enemies in crops (Bianchi *et al*., 2006). Analyzing the surrounding landscape structure, we investigated whether it could affect the abundance of parasitoids in an agroecosystem like the olive orchard. In fact, we found that parasitoid abundance, mainly at family level, was significantly affected by the complexity and composition of some land cover classes, namely ‘Montado’ habitat and vineyards, at a small spatial extent (250 m

radius), and for streams located at higher spatial extent (1000 m radius) around the sites of capture.

Other authors have also observed an increase of parasitoids abundance when analyzing landscape complexity and compositions at small spatial extent (Thies *et al*., 2003; Bianchi *et al*., 2006 and Altieri *et al*., 2005). According to Nicholls et al. (2001), the abundance and diversity of entomophagous insects within a field depends on either the composition of the surrounding vegetation, or the spatial extent of its influence on natural enemy abundance, which in turn is determined by the distance to which natural enemies disperse into the crop.

Variations in temperature and humidity are factors that may also alter the phenology of pests and natural enemies and, therefore, influence insect population growth rate (Logan *et al*., 2003), which might ultimately change the effectiveness of natural enemies in controlling pest abundance from one year to the next (Paredes *et al*., 2013a). Climatic conditions are shown to be very important abiotic variables determining arthropod communities, especially predators (Morris et al., 1999) and parasitoids (Romo & Tylianakis, 2013). Even the presence of pests like *B. oleae* and *P. oleae* are largely affected by temperature (Gkisakis *et al*., 2020; Villa *et al*., 2016).

As for the climatic characterization of the experimental region of our study, and according to data obtained by official meteorological stations during 2016 (IPMA, Instituto Português do Mar e da Atmosfera), the average air temperature (15.91 °C) was in most months higher than the average, in particular, from June to October. The yearly precipitation was classified as normal, but between January and May the values registered were above the average values (991.6 mm). During June to December, only the month of November registered precipitation values slightly above the average (120.1 mm). Overall, the summer was classified as extremely hot and dry.

Our results showed a relation associated with the temperature and rainfall affecting differentially parasitoid presence, at family level. Likely, the five heat waves during the period preceeding the sampling (3 in the summer, 2 in July and 1 in August and 2 in the fall, 1 in September and 1 in October), have had an impact on the abundance and distribution of the observed species.

The differences in abundance of parasitoids found in our sampling compared to other studies, might also been related with a combined effect of climate conditions and composition and quality of ground cover, especially because, in summer season a significant amount of vegetation became dry due to the high temperatures and lack of rainfall registered.

Although the collections were made during short visits during the autumn season and may not represent the full diversity present at a site, several studies surveyed the parasitoid fauna of olives at sites during the course of entire seasons. Studies carried out by Rei (2006) described that the presence of Hymenoptera in the experimental olive groves in Alentejo region was verified in greater number during the second half of June, and this can be partially explained by temperature. The auxiliar entomofauna – parasitoids and predators - is more active from spring on, especially when the population levels of phytophagous start to increase also driven by a thermal adaptation (Amaro, 2003). Furthermore, some authors pointed distinct periods for the main activity of the hymenopteran: in Italy, Viggiani *et al*., (1997) refer to an increased presence of Hymenoptera from June to September, while in Spain, Rodriguéz *et al*., (2012) observed that parasitoids were well represented throughout their sampling period, June to September, although their presence was significantly high during June, July, and August. Other authors mention higher abundances between May and July (Ruano *et al*., 2004, Santos *et al*., 2007 and Álvarez *et al*., 2019) in Portugal and Spain. Also, in Greece, Broumas *et al*., (1973) found that Ichenumonidae maintained an almost constant and uniform presence from spring to autumn, when they began to decrease, while Chalcidoidea populations increased from winter until September.

An important factor in assessing the potential pest control associated with the parasitoid community identified in an ecosystem is their simultaneous presence with the pests. Although the recorded parasitoids were mostly generalists, it was found that late summer/early autumm is a crucial period for the presence of important control agents. Considering the unique temporal nature of the survey, from mid-October to early November, nevertheless, the sampling period coincide with the period when the larvae of the carpophagous generation of the olive moth abandon the fruit to pupate, the black scale is found mainly in the last instars and the olive fruit fly is found mainly in the stages of larva and pupa (Teixeira *et al*., 2000).

For the olive moth, and as observed by Serrano (2016), pest level reduction due to parasitism was more pronounced in the carpophagous generation likely due to *A. fuscicollis*, which shows a great synchronism with its host. At the time of our sampling, the *Prays* adults resulting from the carpophagus generation are likely laying their eggs on the olive leaves (which usually occurs in October/November), giving way to the phylophagous generation. Relevant parasitoids of olive moth, at this stage, should preferencially be parasiting eggs and eventually young instars larvae, and the presence of *Euderus albitarsis*, in the samples, previously associated with the anthophagous generation (Nave *et al*. 2017), is a promossing finding.

Considering the black scale, studies conducted by Tena *et al*., (2007) refer the parasitoid *Metaphycus flavus* numbers peaked at the end of the spring (June) and throughout autumn (October/November), during or shortly after second and third instar black scale occurred in the groves. These instars are the preferred stages for oviposition by *M. flavus*. The presence of parasitized forms of black scale, mostly attacked by *M. helvolus*, was observed from September onwards, as was also reported in Italy by Petacchi & Minnocci (1993). Eventhough we did not register *M. flavus* or *M. helvolus* in our sampling, the Encyrtidae was one of the most represented family (5.62 %) with 17 morphotypes still to be identified. Besides, *Coccophagus lycimnia* a parasitoid of the immature stages of black scale that was abundant during the spring (May/June) and was also abundant in one olive grove in autumn (Tena *et al*. 2007). In our study, several Aphelinidae morphotyopes were found associated with the cover crops, a potentially relevant finding that calls for further research.

In what refers to the olive fruit fly, Boccaccio and Petacci (2009) indicated the presence of two abundant parasitoid species, i.e. *P. agraules* and *E. urozonus*, which are both generalist parasitoids attacking this pest in mid-October. Neueschwander et al. (1983) and Jiménez (1985) reported *E. urozomus* as the most abundant species, especially during the month of August, a time coinciding with the increase of infestation by the olive fruit fly but it gradually decrease with the arrival of autumn and virtually disappeared in late November.

Another hymenopteran of great interest is the Braconidae *P. concolor*, and it is especially in late autumn that this parasitoid can be found spontaneously in the olive groves (Arambourg, 1986). Its presence is linked to recent and historic releases, and according to

Neueschwander *et al*. (1983), reaches the maximum parasitism of *B. oleae* in late November. However, this species was not recorded in our study albeit we specifically looked for its presence. This species is believed to be relatively ineffective as a classical biological control agent in Europe. One reason for its poor performance may be the inability of synchronization between the life cycles of the parasitoid and fly (Clausen, 1978) and the fact that it was not found in the present sampling suggests that in certain geographical areas *P. concolor* is not naturally present in the olive groves and hence not a sympatric natural enemy of the olive fruit fly. However, and despite increasing concerns for the environment and trying to maintain a more natural balance of plants and animals in managed ecosystems, *P. concolor* is still routinely used in the Mediterranean region for inoculative and argumentative releases against the olive fly (Delrio *et al*., 2005). Nevertheless, the possibility of managing the main olive fruit fly solely by a single biological control strategy should be questioned. As confirmed also by other authors (Arambourg, 1986), indigenous Chalcidoidea are poorly active on the first generation of the olive fly, thus allowing it to build a strong biotic potential for the following generations. Also, *P. concolor* parasitizes mainly the third and fourth generations (Arambourg, 1986), thereby not really reducing the final damage to olives. In addition, as late infestations are usually managed via early harvesting, a large proportion of *P. concolor* larvae may be destroyed in the mill (Boccaccio and Petacci, 2009).

From a practical perspective, we aimed to show the presence of a native parasitoid complex in late summer/begin autumn and highlight its importance as a first step in establishing a conservation biological control program against economic relevant pests in olive orchards. Considering the importance of promoting overall functional biodiversity, habitat management through the establishment and maintenance of an ecological infrastructure is essential towards enhancing the effectiveness of natural enemies (Landis *et al*., 2000).

Perspectives towards integrating taxonomic, molecular and ecological data to tackle olive tree pest management

An accurate estimation of parasitoid diversity of crop insect pests is a prerequisite for exploring processes leading to efficient natural biocontrol. More detailed knowledge on their biology and ecology is needed and DNA analyses could be a very usufull tool for the identification of these insects, at any life stages, otherwise often impossible to identify morphologically. DNA barcoding methodologies allow species identification and could also be used in the analyses of insect material collected from the interior of the fruits, to elucidate the lifestyle of the wasps and other insect groups associated with olive trees (Powell *et al*., 2019). A correct taxonomic identification is a critical stage for knowing the parasitoid community and eventually learning on managing it towards limitation of pest species populations.

Moreover, experimentation at fine scales (laboratory or plot level studies) are needed to understand the required ecological factors of specific parasitoid species, and large-scale work will help to place those needs in the context of a specific crop-pest-natural enemy complexes (Gillespie *et all*., 2016).

The ultimate goal is increasing the abundance and diversity creating a suitable ecological infrastructure within the agricultural landscape providing resources and habitat in a way that is spatially and temporally favorable to these parasitoids and practical for farmers to implement.

7. Conclusions

This study has shown the presence of a group of parasitoids which likely play an effective role in the complex trophic web in the olive agroecosystems. Most of the sampled individuals belonged to the superfamilies Chalcidoidea and Icheneumonoidea, referred as important control agents of the main olive pests. The abundance of parasitoids was similar between strata, although a general positive effect of cover crops was observed on parasitoid abundance and diversity.

Weather conditions preceding the captures and landscape heterogeneity may also interact with parasitoids, affecting their population in olive groves. In our work, the

sampling sites were surrounded by different habitat types which could favor the abundance of some parasitoids at a local scale.

The design of the present study consisted in a spot-sampling, which allowed a first step in identifying these parasitoids and raise hypotheses on how weather and landscape effects on that community. A better understanding of the ecology of these parasitoid species is needed, especially concerning space-time dynamics, preferencial and alternative insect host and host plants that support their habitat needs. This would greatly improve our understanding of the complex relationships between natural enemies and their impact on olive pest populations. Longer-term experiments are needed to determine the influence of specific environmental conditions on their prasitoid dynamics and their potential impact on limiting pest populations. The accurate identification of parasitoids is a critical initial step in considering their suitability as a control agent and the use of molecular approaches to complement morphological taxonomic methods for the identification and study of these parasitoids are thus necessary (eventhough not equally efficient between taxa). Further, field trials should be undertaken in order to acquire fundamental knowledge of the biology and requirements of natural enemies and interactions with their hosts as well as their impact on target population and finally to evaluate the effective pest control of these parasitoids.

8. References

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Annex

Figures of the main captured morphospecies.

Figure 11: Aphelinidae sp1.

Figure 12: Aphelinidae sp2.

Figure 13: Aphelinidae sp3.

Figure 14: *Asobara* sp.

Figure 15: Braconidae sp1.

Figure 16: Braconidae sp2

Figure 17: *Chorebus* sp.

Figure 18: Dinotrema sp1.

Figure 19: Dinotrema sp2.

Figure 20: Dinotrema sp3.

Figure 21: *Opius* sp.

Figure 22: Encyrtidae sp1.

Figure 23: *Euderus albitarsis*

Figure 24: *Euplectrus flavipes*

Figure 25: Figitidae sp1.

Figure 26: Figitidae sp2.

Figure 27: Pteromalidae sp1.

Figure 28: Pteromalidae sp2.

Figure 29: Pteromalidae sp3.

Figure 30: Telenomus sp1.

Figure 31: Telenomus sp2.

Figure 32: Telenomus sp3.