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Doctoral Thesis

"Fitness reduction of *Bactrocera oleae* (Rossi) due to the alteration of the endosymbiotic bacteria titer"

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1. INTRODUCTION

1.1 *BACTROCERA OLEAE*: THE KEY PEST OF *OLEA EUROPAEA*

1.1.1 HISTORY AND ORIGIN OF THE SPECIES

The olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae) (Rossi, 1790) was first observed and described in the human history by the ancient Greek academic, Theophrastus of Eresus (c. 371–287 BC), colleague and successor of Aristotle in the Peripatetic school (Fortenbaugh and Gutas, 1992). Between c. 350 BC and c. 287 BC, Theophrastus wrote the *Historia Plantarum*, one of the most significant contributions to botanical science during ancient times and the Middle Ages, in which he mentioned the presence of a worm into the olive fruit and underlined the detrimental effect of the insect on the olive oil production (Hort, 1916).

> "Now the worm which infests the olive, if it appears below the skin, destroys the fruit; but if it devours the stone it is beneficial. And it is prevented from appearing under the skin if there is rain after the rising of Arcturus.
>
> Worms also occur in the fruit which ripens on the tree, and these are more harmful as affecting the yield of oil.
>
> Indeed these worms seem to be altogether rotten; wherefore they appear when there is a south wind and particularly in damp places."
>
> Theophrastus of Eresus, *Historia Plantarum*, IV volume, XIV, 9-10

The origin and ancient history of the olive fruit fly are strictly linked to its host plant: the olive tree, *Olea europaea* (Nardi *et al.*, 2005; 2010). It is due to their exclusive relationship and coevolution that indicates a long-term and constant plant-insect association (Nardi *et al.*, 2010). Thus, it might be hypothesized that the evolution and the historical distribution of *B. oleae* are

strongly related to the evolutionary process and the range expansion of its host plant (Nardi *et al.*, 2010).

Whereas these species are commonly associated to the Mediterranean Basin, where the olive production has been historically and economically of great importance, neither olive tree nor olive fruit fly originated in this region (Nardi *et al.*, 2005; 2010). Their common origin is still an issue for the scientific community but recent studies indicate that both the species have been originated in Southern-Eastern African countries (Zohary 1994; Angiolillo *et al.* 1999; Nardi *et al.*, 2005; 2010).

The basal diversification of the olive tree probably took place in consequence of the desertification of African midlands during the early Pliocene with the division of two lineages: the Asian and African lineages of *O. europaea* subsp. *cuspidata* and the European and North African *O. europaea* subspecies group (Nardi *et al.*, 2010).

Before the domestication, since Pre-Quaternary, the original and dominant form of the olive tree in the Mediterranean region was the wild olive tree, *O. europaea* subsp. *cuspidata* var. *sylvestris* (Besnard *et al.*, 2007; 2009; Baldoni *et al.*, 2002 Zohary and Spiegel-Roy, 1975) that have been later mainly replaced by the cultivated form (Lumaret *et al.*, 2004). In spite of the reduced pulp and oil content of their fruits, wild olive trees have been utilized by humans in the Neolithic (Zohary and Hopf, 2000) and they had represented the sole host plant of *B. oleae* (Katsoyannos, 1992; Tzanakakis, 2006).

The expansion of the olive fruit fly in the Mediterranean Basin probably took place during Pleistocenic recolonization when the basal diversification of the African and Mediterranean populations of *B. oleae* occurred (Nardi *et al.*, 2010). Thus, the Mediterranean population was first present on the wild olive form (Nardi *et al.*, 2010).

Domestication of the wild variety probably occurred in the Middle East during the 4th millennium BC (Lumaret *et al.*, 2004; Zohary and Hopf, 2000) and subsequently, cultivated olive tree, *O. europaea* subsp. *europaea* var. *europaea*, have been introduced in the Mediterranean region through human-mediated commercial exchanges (Ruiz Castro, 1948; Boardman, 1976). Indeed, the Mediterranean population of the olive fruit fly was first present on wild olive and subsequently, after the olive tree domestication, transferred from its original host to a richer food source provided by cultivated olive form, with a significant adaptation process in host searching and feeding mechanisms (Nardi *et al.*, 2010).

1.1.2 GEOGRAPHIC RANGE

Nowadays, the olive fruit fly is wide-spread (Figure 1), mainly covering the natural geographic range of the wild and cultivated olive tree with rare exceptions in isolated areas or where low temperatures restrict its development (Tzanakakis, 2006). It is found in all the Mediterranean country of Europe, Asia, and North Africa, in the Canary Islands, in South and Central Africa, in the Middle East and Pakistan (Nardi *et al.*, 2005; Daane and Johnson, 2010). Recently, it has been accidentally introduced to California (USA) and Mexico (Rice, 1999; Rice *et al.* 2003; Nardi *et al.*, 2005).

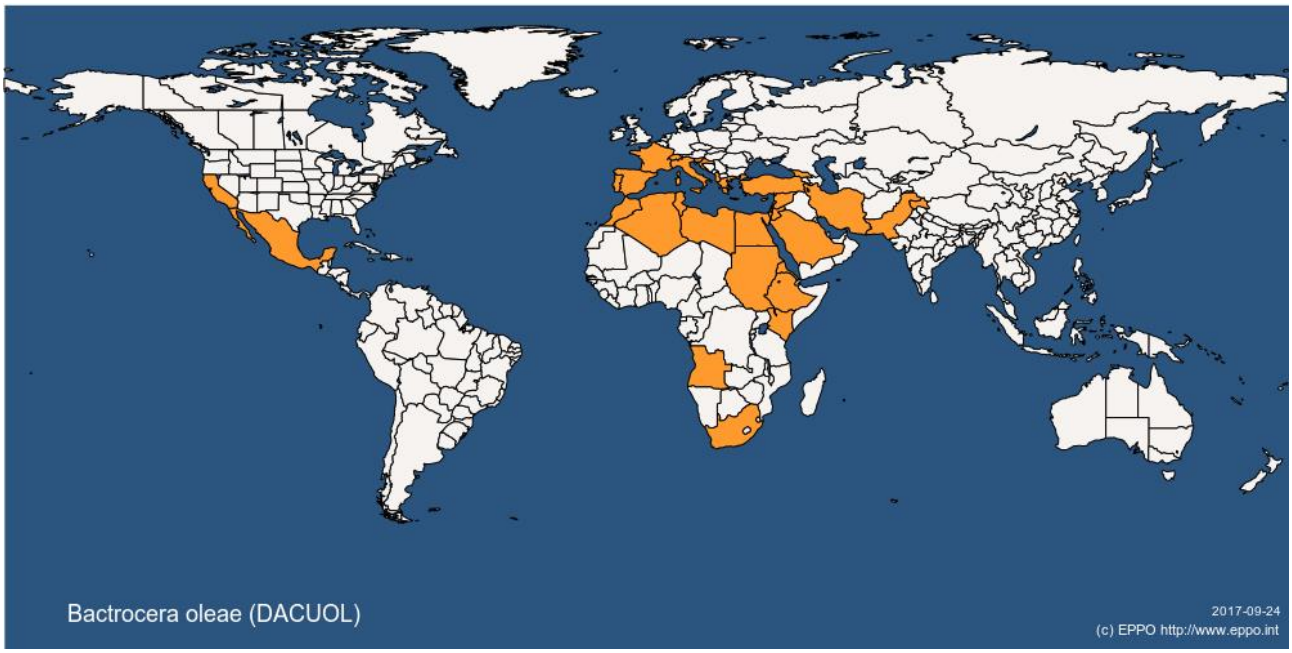

Figure 1: B. oleae world geographical distribution. Last updated: 2016-07-04

1.1.3 TAXONOMY AND MORPHOLOGY

B. oleae (Diptera: Tephritidae) belongs to the Dacinae subfamily (Table 1) that includes carpophagous species in which the olive fruit fly is the only exponent of the *Bactrocera* genus inhabiting the Mediterranean basin.

Table 1: Taxonomical classification of B. oleae

Taxonomical classification	
Kingdom:	Animalia
Phylum:	Arthropoda
Class:	Insecta
Order:	Diptera
Suborder:	Brachycera
Section:	Schizophora
Subsection:	Acalyptrata
Superfamily:	Tephritoidea
Family:	Tephritidae
Subfamily:	Dacinae
Genus:	*Bactrocera*
Subgenus:	*Daculus*
Species:	*B. oleae* (Rossi, 1790)

Adults: 4-5mm in length; head characterized by fulvous face with a pair of black spots under the antennae, brownish maxillary palpi and mouthparts and blue-greenish compound eyes; thorax orange-reddish with a black scutum and metanotum, yellowish postpronotal lobes and an episternal stripe not reaching anterior notopleural seta; scutellum pale yellow and black post-scutellum. Wings are hyaline with stigma, veins and a small spot around the apex of vein R4+5 fuscous; abdomen mainly fulvous with dark brown to black anterolateral corners on tergites III and IV and black spots on tergites I to IV. Female is characterized by an evident serrated ovipositor, partly invaginated in urite VII; male tergite III with a pecten (setal comb) on each side; surstylus with short posterior lobe (Belcari, 1989; Tremblay, 1994).

Eggs: elliptical and whitish, typically with a length of about 0.7 mm and a diameter of 0.12 mm, they present a mircopilum that is used to carry out the respiratory functions (Mouzaki and Margaritis 1987; Mouzaki *et al.*, 1991).

Larvae: acephalic with a greatly reduced head, completely enclosed in the thorax. It remains a retractile capsule that bears strongly modified mouthparts transformed into an apparatus called cephalopharyngeal skeleton (Phillips, 1946; Belcari, 1989; Tremblay, 1994).

There are three larval instars that can be distinguished by shape, size of the mouthparts and the presence of the thoracic stigma. The color is whitish to yellowish and they present a caudal ridge. The first larval instar has the mouthparts barely sclerotized with an apical unculus and shows a metapneustic respiratory system. The second larval instar has partially sclerotized mouthparts and shows an amphipneustic respiratory system with characteristic prothoracic stigma. The third larval instar is provided with a fully developed cephalopharyngeal skeleton and has an amphipneustic respiratory system with fully developed prothoracic stigma. The full-grown larva reaches 6-7 mm long and is able to jump; it presents dorsal spinules on segment T1-A1 (Phillips, 1946; Belcari, 1989; Tremblay, 1994).

The puparium is 4-6 mm long with an elliptical shape, normally yellowish to brownish with markedly evident segments (Tremblay, 1994).

1.1.4 LIFE CYCLE AND BIOLOGY

The olive fruit fly is considered a homodynamic pest since it can reproduce and develop during all the year if temperature and humidity are favorable (Tzanakakis, 2003; 2006). A limiting factor for its development is the availability of olive fruit since the larval development only occurs in the host mesocarp (Fletcher, 1987). In fact, *B. oleae* has different feeding behaviors depending on the life stage: adult fly is polyphagous feeding on several organic sources whereas larval stages are monophagous feeding exclusively on the fruit pulp of *O. europaea subsp. europaea (*cultivated and wild forms) and *O. europaea subsp. cuspidata* (Fletcher, 1987; Daane and Johnson 2010).

To guarantee access to food sources to the new hatching larvae, *B. oleae* females perform a puncture on the olive fruit surface by their ovipositor and lay their eggs in the olive pulp, beneath the olive skin (Tzanakakis, 2003). Ovipositing females are increasingly attracted by olive until the fruit reached 3.5 g in size. From this stage, the preference for unripe green fruits is stable while the preference for ripe black fruit start to decline (Daane and Johnson 2010)

As soon as the larva emerges, it immediately starts to feed on the olive pulp forming galleries into the mesocarp that progressively increase their size and deepens its path into the endocarp during the larval development. At the end of the third instar, the larva moves from the proximity of the endocarp to the olive surface and create a pupation room right beneath the olive skin where it performs a hole that may serve as air duct, if the pupation takes place into the olive, or

as an exit hole for the larva itself, if the pupation occurs in the soil (Tzanakakis, 2006). Usually, *B. oleae* larvae have a tendency to pupate into the olive pulp during the summer whereas in autumn they prefer to pupate in the soil to a depth of 1-3cm (Tzanakakis, 2003).

The length of each developmental stage is deeply influenced by temperature and environmental condition. At optimal conditions the egg incubation lasts 1-2 days, the larval development 8-9 days and the pupal development 9-10 days, ranging from 1 to 19. From 8 to 37 and from 9 to 49 respectively, under different temperature regimes (Neuenschwander and Michelakis, 1979; Tsitsipis, 1977). The lower and upper-temperature threshold are respectively 7.5-10°C and 30-32°C for egg development and 10-12.5°C and 30-32°C for larval development, with an optimal temperature at 24-25°C (Tsitsipis, 1977). Even if the pre-imaginal development can be achieved at 30°C, in this condition the mortality is high with pupal death up to 48% (Tsitsipis, 1980).

After the pupation the new adult emerges and completes gonad maturation in 5-8 days, reaching the sexual maturity (Canale *et al.*, 2012). *B. oleae* females are oligogamous and generally mate 1-3 times during their life cycle (Zouros and Krimbas, 1970) although males are polygamous and can mate daily depending only on receptive female's availability (Zervas, 1982). A daily rhythm in sexual activities has been observed since matings take place in late afternoon and at dusk, both in laboratory and field conditions (Mazomenos, 1989). Females start the oviposition 3-5 days after mating, laying from 1 to 10 eggs per day (Tzanakakis, 2006).

The number of annual generations in field conditions is dependent on several factors such as environmental temperature, humidity, microclimate within the olive orchard and the quality and availability of host fruits (Burrack and Zalom, 2008; Kounatidis *et al.*, 2008). Thus, the recorded number of annual generations widely varies within the olive fruit fly geographical range that includes several climatic regions (Daane and Johnson, 2010). In Southern Italy, within the coastal zone, the warm climate conditions allow the development of 5-7 generations per season while in Central and Northern Italy, characterized by colder winters, the annual generations are generally 3-4 (Delrio and Cavalloro, 1977).

In Italy as in most of the Mediterranean countries, *B. oleae* seems to be best adapted to autumn period conditions, in which the larval development is enhanced by the wide availability of olive fruits, high humidity and mild temperatures with maximum values that infrequently reach 27°C. Thus from mid-September until mid-November, the population density reaches the highest level (Tzanakakis, 2006; Wang *et al.*, 2009). The lower population observed in summer is probably due to the reproductive quiescence of the female that has been hypothesized to be caused by the

high temperatures and low humidity (Fletcher and Kapatos, 1983; Fletcher *et al.*, 1978; Mourikis and Fytizas, 1970).

Several studies have tried to address the question about how the olive fruit fly overwinters and so far, it has been speculated that it can overwinter as an adult, as larvae in the host fruit or as pupae in the soil (Kapatos and Fletcher 1984; Neuenschwander *et al.* 1981). The population dynamics during winter and spring seems to be complex (Raspi *et al.* 1997. 2002; Ragaglini *et al.* 2005) but the more accredited hypothesis is that the *B. oleae* adults observed in early spring derive from the previous autumn population (Ragaglini *et al.*, 2005). A recent study on the overwintering population reported that, in March and April, females ovaries contains follicular relicts and sperm cells indicating the occurrence of egg fertilization and demonstrating that the overwintering adults complete one generation in early spring (Marchini *et al.*, 2017).

1.1.5 DAMAGE

Depending on the yearly fluctuations of the climate conditions, the olive fruit fly may cause severe damage and economic losses in the olive production due to yield reduction and degradation of products composition, quality, and properties (Pereira *et al.*, 2004). The impact of this pest on the olive oil and olive table sector is frequently extremely detrimental and the average annual loss caused by olive fruit fly infestation is estimated to be 5% of the total world production with an estimated economic loss of 800 million US$ a year (Montiel Bueno and Jones, 2002). In Italy, during the 2014-2015 season in which the olive fly infestation was very high, a decrease of 35% in olive oil production in comparison with the previous season has been recorded (source: ISTAT and ISMEA).

B. oleae may affect the olive production in numerous ways (Daane and Johnson 2010). Immature fruit may undergo to abortion if stung during the early development but the main damage is certainly caused by the larval stages during development as they chew and feed on the olive pulp destroying it and in some cases determining a premature fruit drop (Tzanakakis, 2006). Therefore, the quantitative loss of the yield is a direct effect of the larval feeding while microorganisms' proliferation in the feeding tunnels and necrotic areas occurrence that affect the fruit quality are indirect effects (Angerosa *et al.*, 1992).

It has been estimated that a single larva can consume from 50 to 150 mg of olive fruit pulp during its development depending on cultivar (Neuenschwander and Michelakis, 1978). The

impact on crops of the larval development widely varies with the final use of the fruit. Table olive crops present a tolerance threshold of *B. oleae* infestation near zero larvae per fruit while the threshold values are much higher in the cultivation of oil olive crops (e.g., 10–30% of infested fruits) (Daane *et al.*, 2005; Neuenschwander and Michelakis, 1978).

The overall impact of olive fruit fly on olive oil production is influenced by several factors such as olive cultivar, harvest date, length of storage time before pressing, the presence of microflora and the timing and severity of the pest infestation (Torres-Villa *et al.*, 2003; Tzanakakis, 2006; Tamendjari *et al.*, 2004; Pereira *et al.*, 2004). *B. oleae* larval infestation increases olive oil acidity and the alteration of phenol, peroxides and sterol content that significantly reduce the oil quality with the occurrence of musty off-flavors and the consequent downgrading of the product (Gómez-Caravaca *et al.*, 2008; Angerosa *et al.*, 1992). The increase of oil acidity and the consequent decrease of olive oil quality if is directly proportional to the length of the fruit storage and to the presence of microorganisms including bacteria (*Xanthomonas*), yeast (mainly *Torulopsis* and *Candida*) and molds (mostly *Fusarium* and *Penicillium*) that thus contributes to the oil degradation (Torres-Villa *et al.*, 2003).

B. oleae has shown to differentially infest olive crops depending on the cultivars, moreover there are cultivars that have a higher susceptibility to the infestation, and thus, cultivars can also deeply influence the pest impact on the crop (Gumusay *et al.*, 1990; Alzaghal and Mustafa, 1987; Burrack and Zalom, 2008). The infestation rate can range from less than 10% to up to 31% depending on the olive tree cultivar (Iannotta and Scalercio, 2012) thus, the cultivar can deeply influence the olive oil quality since an infestation rate of 10% can still lead to a high quality olive oil (Gucci *et al.*, 2012).

The mechanisms on which the different level of tolerance to *B. oleae* infestation is based is complex and may rely on chemical factors, as the oleuropein and cyanidine abundance into the olive pulp, mechanical barriers, as the aliphatic waxes presence on the esocarp, and morphological characteristics of the fruits (Corrado *et al.*, 2016; Grasso *et al.*, 2017).

The molecular response of the olive fruit to larva feeding have been also investigated and the tolerance mechanism seems to involve a large variety of genes with a known role in oxidative stresses response, defense, plant structure and metabolism (Grasso *et al.*, 2017).

Other factors that may influence the pest impact are the phenological stage of the crop, fruit size, color and weight, epicarp hardness and surface covering and in particular the amount of aliphatic

waxes present (Donia *et al.*, 1971; Iannotta *et al.* 2007. Neuenschwander *et al.*, 1985). It seems that high amounts of oleuropein and cyanidine decrease *B. oleae* infestations (Iannotta *et al.* 2007).

1.1.6 CONTROL STRATEGIES

During the last 40 years, the control strategies of *B. oleae* have been based on the wide use of organophosphate insecticides such as dimethoate and fenthion generally utilized as bait sprays and cover sprays (Skouras *et al.*, 2007; Margaritopoulos *et al.*, 2008; Kakani and Mathiopoulos, 2008). More recently, pyrethroids and spinosad have been introduced into the chemical pest management of the olive fruit fly (Margaritopoulos *et al.*, 2008; Thomas and Mangan, 2005).

Since 1970, it is well known that *B. oleae* may develop resistance to dimethoate and other organophosphates (Tsakas and Krimbas, 1970; Stasinakis *et al.*, 2001. Skouras *et al.*, 2007). Thus, the occurrence of insecticides resistance has driven the scientific community to research alternatives to organophosphate cover sprays like mass trapping programs, adopted since 1985, that mainly rely on attraction to baits, colors and/or pheromones (Broumas, 1985).

Fruit flies are commonly attracted to wide varieties of compounds (Dìaz-Fleischer and Aluja, 2001) such as molasses, protein hydrolysates, yeast, NuLure and ammonia-releasing salts, which have been utilized as baits in several trapping strategies for the olive fly pest management (Haniotakis *et al.*, 1986; Katsoyannos and Kouloussis, 2001; Thomas and Mangan, 2005). The color yellow is highly attractive to *B. oleae* adults thus, it has been integrated into the design of numerous kinds of sticky and McPhail traps used either for mass trapping or monitoring (Katsoyannos, 1989). Nonetheless, yellow traps usually catch several beneficial insects and for this detrimental effect on the biodiversity of the olive tree canopy, this control strategy has been abandoned in some regions (Neuenschwander, 1982).

Between 1977 and 1980. the sex pheromones produced by male and female have been discovered, synthesized and then applied to the trapping control strategy with a great improvement of its efficacy and selectivity (Haniotakis *et al.*, 1977; Baker *et al.*, 1980. Daane and Johnson, 2010).

As evidence underlined that *B. oleae* wild populations may potentially develop noteworthy levels of insecticides resistance (Tsakas and Krimbas, 1970; Stasinakis *et al.*, 2001. Skouras *et al.*,

2007), traditional control strategies, mainly provided with cover and bait sprays, may lose their efficacy in the near future (Daane and Johnson 2010). Therefore, the development of noninsecticidal control strategies is essential to avoid the resistance occurrence reducing the selection pressure on the field populations (Daane and Johnson 2010). Furthermore, alternative control methods to chemical pesticides may significantly reduce the environmental impact of olive crop and guarantee a safer product for consumers (Saour and Makee, 2004).

Since the 1970s, the sterile insect technique (SIT) has been explored as an insecticide-free method on olive fruit fly control without effective results (Economopoulos 1972; Economopoulos and Zervas 1982; Estes *et al.*, 2012b). In fact, gamma-sterilized, laboratory-reared males have markedly shown a reduced longevity and ability to search, find and mate with wild females (Economopoulos, 2002). Fitness decrease of laboratory-reared fly is probably due to the alteration of the fly's bacterial microbiota, induced by antibiotics administration into the artificial diet, and particularly to the elimination of olive fruit fly's specific endosymbiont, "*Candidatus* Erwinia dacicola", which deeply increase the adult and larval fitness of this pest (Estes *et al.*, 2012b; Ben-Yosef *et al.*, 2010; 2014; 2015; Ras *et al.*, 2017).

Another alternative to synthetic chemical pesticides in *B. oleae* pest management is the kaolin-based particle film application. The product, which is obtained by a high rafination of the kaolinic mineral, is sprayed onto the foliage in a liquid suspension and once the water evaporates a white and powdery film remains on the leaves and fruits surface. The insecticidal properties of this product are not due to the toxic effect on the insect pest but to a combination of its repellent nature, antiovipositional qualities and the high reflectiveness of its white coating, which could severely damage olive fruit fly orientation within the orchard (Saour and Makee, 2004). The application of this product in olive groves have shown a great potential for the replacement of chemical pesticides since the decrease of the infestation level obtained is comparable with treatments with conventional insecticides such as Dimethoate (Saour and Makee, 2004).

Copper products are also successfully used in olive groves for the *B. oleae* control (Belcari and Bobbio, 1999; Sacchetti *et al.*, 2004; Iannotta, 2004; Caleca *et al.*, 2010). Copper spray application leads to a significant decrease of the infestation and interestingly determines a high rate of larval mortality, suggesting that the treatment may act as a symbioticides (Rosi *et al.*, 2007). It was also demonstrated that copper products could act as an adult oviposition deterrent (Prophetou-Athanasiadou *et al.*, 1991).

Biological control has also been attempted numerous times throughout the twentieth century. Most of the trials resulted unsuccessful because of the transportation difficulties of the natural enemies, their lack of adaptability to the new propagation environments and / or cultivated varieties, and the rearing technical hitches concerning both the olive fruit fly and parasitoids (Delucchi V. 1957; Monastero and Delanoue, 1966; Kapatos *et al.*, 1977; Liaropoulos *et al.*, 1977; Wharton, 1989; Raspi and Loni, 1994; Miranda *et al.*, 2008; Yokoyama *et al.*, 2008).

1.2 ENDOSYMBIOSIS BETWEEN BACTERIA AND *B. OLEAE*

1.2.1 ENDOSYMBIOTIC BACTERIA WITHIN INSECTS

The mutualistic symbiosis between prokaryotes and eukaryotes has significantly contributed to the evolution of life on Earth (Margulis and Fester, 1991). In contrast with most of the unicellular organisms, eukaryotes have limited capabilities in synthetizing metabolic compounds; therefore, symbiosis with prokaryotes has delivered an evolutionary strategy to increase the range of metabolic sources. One of the best examples of eukaryotic-prokaryotic symbiosis is mutualism between insect and bacteria.

Insects are the most abundant and evolutionary successful class among the Animalia Kingdom (Novotny *et al.*, 2002). This is reflected in the variety of habitats in which they live and in the incredible amount of different species which is estimated to be more than 4 million. This success can be explained by the multiplicity of feeding strategies they exploit (Schoonhoven *et al.*, 2005; Slansky and Rodriguez, 1987). The extraordinary capacity to adapt to a vast variety of ecological niches is frequently linked to the association with symbiotic microorganisms (Douglas, 2009; Ratzka *et al.*; 2012) and it has been hypothesized that the large diversity of insect species can be due to their tendency to associate with beneficial bacteria (Janson *et al.* 2008).

The early associations between insects and bacteria, estimated about 300 Million Years ago (Moran and Telang, 1998), considering the several advantages that bacteria offer to their insect hosts, could be the key factors in the evolutionary success of this class of organisms (Douglas, 1998; Moran and Bauman, 2000).

It has been estimated that more than 15–20% of all insect species live in symbiotic relationships with bacteria (Buchner, 1965). These ancient associations, known since last century by Petri (1909), allow hosts to achieve new niches hence contributing to host diversification and success (Wernegreen *et al.*, 2002).

During the past 20 years, technological advances in molecular characterization and the advent of the Next Generation Sequencing techniques have allowed exploration of the world of these symbionts, which are mostly uncultivable bacteria (Moran and Wernegreen, 2000).

Endosymbiotic bacteria have recently become the subject of several scientific investigations, which have explored their diverse ecological and evolutionary effects on the insect host. These effects include host fitness advantages such as nutritional improvement (Ben-Yosef *et al.*, 2015;

Brune and Dietrich, 2015; Douglas, 1998), enhancement of insect metabolism (Douglas, 2013; Zientz *et al.*, 2004), thermal tolerance (Dunbar *et al.*, 2007), upgrading of parasitoids and/or pathogen resistance (Kaltenpoth, 2009; Currie *et al.*, 2003) and manipulation of plant physiology (Giron *et al.* 2016b). Furthermore, they can affect the host reproductive system, influencing the reproductive isolation, population structure, and speciation (Moran *et al.*, 2008).

While the insect host may obtain several advantages during their life history thanks to the relationship with beneficial bacteria, the endosymbiotic bacteria can benefit from a reasonably constant environment within the insect body and from a reliable transmission to host offspring (Klepzig *et al.* 2009).

Endosymbiotic bacteria are generally classified into primary and secondary symbionts. Primary endosymbionts are mostly intracellular and inherited maternally via the germline (Giron *et al.*, 2016a). These symbionts have an obligate and long-term association with their insect host and often live in specialized host cells (so-called bacteriocytes) that are essential for the host's nutrition, survival and reproduction (Baumann, 2005; Braendle *et al.*, 2003; Tremblay, 1960). One of the most intensively studied examples of primary endosymbionts is *Buchnera aphidicola* (Munson *et al.*, 1991), which ensures the adaptation to feeding on phloem to aphid hosts.

Conversely, many endosymbionts, which can be found intra- or extracellularly, do not reside into the bacteriocytes and are facultative for host survival and reproduction (Dale and Moran, 2006). These secondary endosymbionts may be both vertically and horizontally transmitted (Olivier *et al.*, 2010; Dale and Moran, 2006; Russell *et al.*, 2003) and, even if they are not required for the host's development, they may deeply influence their host biology (Giron *et al.*, 2016a). It is the case of *Wolbachia*, one of most widespread endosymbiont among the insect class, well known for its impact on host reproduction (Engelstädter and Hurst, 2009) but also for conferring resistance to several virus infections (Joubert and O'Neill, 2017) and for mediating the interactions between its insect hosts and the plant they consume (Kaiser *et al.*, 2010; Giron and Glevarec, 2014).

It is well known that the majority of insect species which harbor beneficial bacteria are monophagous and hemimetabolous (Baumann, 2005; Baumann and Moran 1997; Gil *et al.* 2004; Kikuchi *et al.* 2009). In fact, many of these hosts feed on unbalanced diets that lack nitrogen, essential amino acids, and vitamins, which are required for basic metabolic needs and provided by their microbial partners (Douglas 2009. Moran *et al.*, 2008). Within the Homoptera order, there are several examples of endosymbiotic bacteria, which synthesize the nutrients that lack in

their host's diet (Douglas *et al.* 2001; Moran and Degnan 2006). In contrast, few polyphagous insects have been studied for mutualistic endosymbiosis (Estes, 2009). Equally, only a few holometabolous insect are known to harbor vertically transmitted and permanent endosymbiotic bacteria since the tissues where they would reside may be degraded during metamorphosis (Estes, 2009).

A particular case study of polyphagous and holometabolous insect species permanently associated with a specific beneficial bacterium is the olive fruit fly, *B. oleae,* around which this thesis is focused on.

1.2.2 ENDOSYMBIOTIC BACTERIA WITHIN TEPHRITIDS

The Tephritidae family is one of the largest families of the Diptera order, with more than 4710 of described species and almost 500 genera (White, 2006; Pape and Thompson, 2017). Within tephritids, just about 70 species are considered as important agricultural pests (White and Elson-Harris, 1992) and some of the most detrimental genera, such as *Anastrepha, Ceratitis, Bactrocera, Dacus, Rhagoletis* and *Toxotrypana* mainly infest fruits (Norrbom *et al.*, 1999). Nevertheless, there are considerable differences in feeding strategy among the developmental stages of these species. The larval stages of Dacinae and Trypetinae subfamilies predominantly use fleshy fruits of host plants of a large variety of species as the food source while the Tephritine subfamily counterparts feed on plant tissue and mainly on the flowerheads of Asteraceae species (Headrick and Goeden, 1998). In contrast, adult fruit flies are generally polyphagous and exploit various substrates found on fruit and foliar surfaces of which plant exudates, homopteran honeydews, and bird droppings are considered the primary resources providing carbohydrates and nitrogen (Bateman, 1972; Fletcher, 1987. Drew and Yuval, 2000).

Shreds of evidence of hereditary endosymbiotic bacteria in Tephritidae family have been first described in the olive fly *B. oleae,* by the Italian plant pathologist Lionello Petri (Petri, 1909), who reported an evagination of the foregut, a cephalic organ connected to the pharynx of the adult fly, so-called oesophageal bulb, in which symbiotic bacteria were harbored. The presence of the oesophageal bulb has been further detected in all adults' tephritid flies even with different shapes and sizes from *B. oleae* ones and not always linked to the presence of endosymbiotic bacteria (Girolami, 1973. 1983).

Girolami (1973) described four principal types of oesophageal bulbs in the Tephritidae family based on morphological and histological observations and on the presence or absence of bacteria within (Figure 2):

1. "*Bactrocera* or *Dacus* type," characteristic of *B. oleae* according to the description of Petri (1909). It is large and sphere-shaped, with a base provided with columnar epithelial cells and full of symbiotic bacteria.
2. "*Ceratitis* type," characteristic of *Ceratitis capitata* and other species of the subfamilies Trypetinae and Dacinae (except for *B. oleae*). This type is also spherical but smaller than the first type, frequently containing bacteria and with a columnar epithelium in the apex.
3. "*Ensina* type," characteristic of some genera of the subfamily Tephritinae (*Acanthiophilus, Trupanea, Ensina, Noeeta, Tephritis, Urophora, Xyphosia, Campiglossa, Oxyna, Sphenella*). It is small and ovoid-shaped, with a wide muscle tunic and without columnar epithelium in the apex. Bacteria have been never detected in this type of oesophageal bulb. Remarkably, all the Tephritinae species in which symbiotic bacteria have been found in a specific tract of the midgut present this type of esophageal bulb.
4. *"Chaetorellia* type" characteristic of other Tephritinae species (*Chaetorellia, Chaetostomella, Orellia, Terellia*). This type is an intermediate oesophageal bulb, between the "*Ceratitis* type" and the "*Ensina* type".

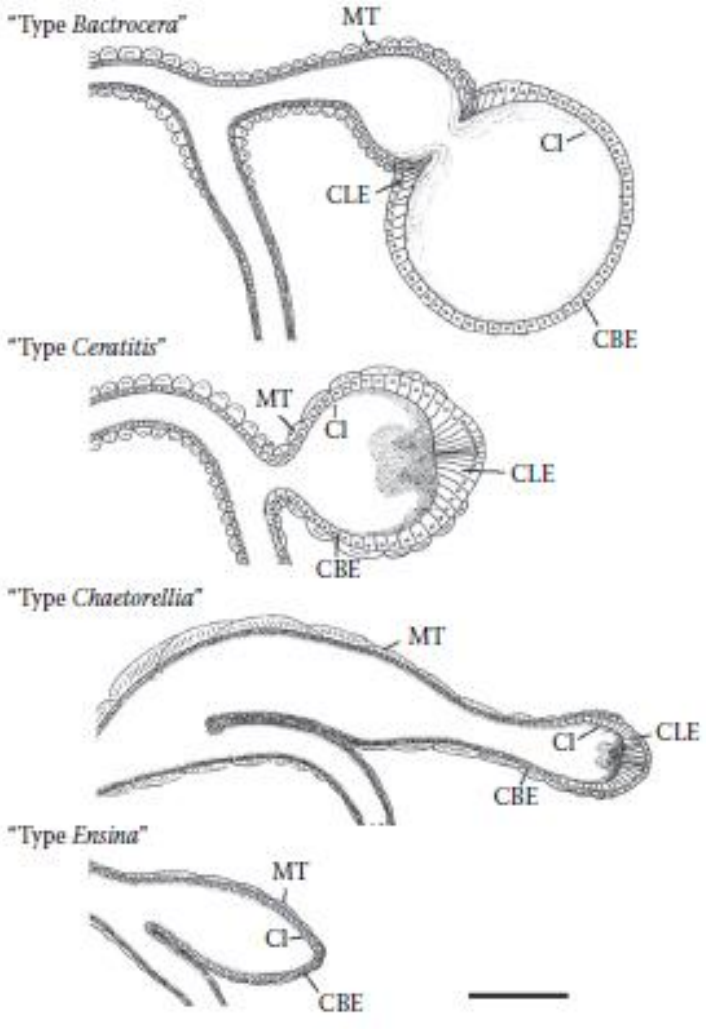

Figure 2: Different types of oesophageal bulbs of tephritid species. Abbreviations: CBE, cuboidal epithelium; CLE columnar epithelium; CI, cuticular intima; MT, muscle tunic. Bar = 0.1mm. (From Girolami V., Redia, 54. 269-294. 1973).

In the case of flies belonging to the subfamily Tephritinae, German zoologist Hans-Jürgen Stammer (Stammer, 1929) first reported the presence of symbiotic bacteria in several genera. Subsequent studies have proposed a specific genus of endosymbiotic bacteria "*Candidatus* Stammerula" to be associated with several species of the subfamily Tephritinae. In particular, "*Ca.* Stammerula tephritis", predominantly in association with the genus *Tephritis* and "*Ca.* Stammerula trupanea" which include symbionts of the *Trupanea*, *Phaeogramma* and *Capitites* genera (Mazzon *et al.*, 2008; Mazzon *et al.*, 2010; Mazzon *et al.*, 2011; Viale *et al.*, 2015). These unculturable bacteria belong to the family Enterobacteriaceae seem to play an important nutritional role in the adult fly fitness (Mazzon *et al.*, 2008; Mazzon *et al.*, 2010; Mazzon *et al.*, 2011; Viale *et al.*, 2015). These symbionts are vertically transmitted from the mother to the offspring but, in contrast with the case of *B. oleae.* Several studies affirm that in the adult flies of

the Tephritinae subfamily, the esophageal bulb seems to be empty of symbiotic bacteria that are indeed located in the gut lumen, externally to the peritrophic membrane in contact with midgut epithelial cells (Mazzon *et al.*, 2008; Mazzon *et al.*, 2010; Mazzon *et al.*, 2011; Girolami, 1973. 1983).

Olive fruit fly and Tephritinae subfamily flies have been studied for their tight association with beneficial bacteria and it has been hypothesized a potential co-evolutionary interaction between these bacteria and its host (Mazzon *et al.*, 2008. 2010; Estes *et al.*, 2009).

Tephritids are largely known to harbor also secondary endosymbiotic bacteria of which numerous belong to Enterobacteriaceae family such as *Enterobacter, Pantoea, Citrobacter, Providencia* and *Klebsiella* genera that consistently inhabit the tephritid gut (Lauzon *et al.*, 1998; 2003; Liu *et al.*, 2016). Most of these free-living, rot-inducing bacterial genera are commonly inoculated into the fruit by females during oviposition and improve larval nutrition participating in the carbon and nitrogen cycle of the host with the synthesis of essential amino acids and minerals (Miyazaki *et al.*, 1968; Lauzon *et al.*, 2000; 2003; Lauzon, 2003; Drew and Lloyd, 1991; Behar *et al.*, 2008; 2009).

In *Ceratitis capitata* (Wiedemann), intestinal associated Enterobacteria have shown to be involved in nitrogen fixation and in pectin degradation and to prevent the colonization of pathogenic bacteria thus, improving the host fitness (Behar *et al.*, 2005; 2008)

Lauzon *et al.* (2000) demonstrated that most of the Enterobacteria species associated with tephritids form a biofilm into the adult gut that contributes in the catabolism of nitrogen and plays an important role in adult flies survival in nature. Biofilms are metabolically active structures (Costerton *et al.* 1995) that frequently work as a coordinated digestive organ with complex and diverse metabolic capabilities. The gut biofilm, in fact, can also catabolize several toxic compounds such as the plant antifeeding substances. This is the case of *E. agglomerans*, an endosymbiont of *Rhagoletis pomonella* that has been proven to degrade phloridzin, a toxic and antifeeding compound of *Malus* spp. (Lauzon *et al.*, 2003). Therefore, the architecture and width of the biofilm may regulate the transit time of nutrients into the gut allowing a maximized digestion and assumption of them and at the same time may act as an effective barrier for toxic substances (Gjersing *et al.* 2005. Lauzon *et al.*, 2009).

As it is well known that some symbiotic bacteria mediate detoxification of insect food sources (Cejanavarro *et al.*, 2015; Genta *et al.*, 2006), there are also evidence that they can degrade

pesticides conferring insecticides resistance to their hosts (Boush and Matsumura, 1967; Kikuchi *et al.*, 2012). It is the case of one of *Bactrocera dorsalis* gut symbionts, *Citrobacter freundii*, which is able to degrade trichlorphon, a moderately toxic organophosphate insecticide (Cheng *et al.*, 2017). The *B. dorsalis* populations that harbor *C. freundii* in their gut have shown a prevalent resistance to this insecticide treatment (Cheng *et al.*, 2017).

Considering the digestive physiology of Diptera Cyclorrhapha (Lemos and Terra, 1991; Terra and Ferreira, 2012) and the attributes of symbiosis across the Tephritidae (Girolami, 1973; Martinez-Sanudo, 2009), it has also been hypothesized that fruit flies might satisfy their protein needs by digesting symbiotic bacteria which are previously collected in their gut (Drew *et al.*, 1983; Drew and Yuval, 2000).

Another important example of facultative tephritid endosymbiont is represented by *Wolbachia pipientis*, an obligatory, maternally inherited, intracellular, endosymbiotic bacterium that is probably the most widespread bacterium infecting insect species all over the world (Apostolaki *et al.*, 2011). This bacterium is an alpha-proteobacteria of the Rickettsiaceae family (O'Neill *et al.* 1992; Werren 1997; Stouthamer *et al.* 1999; Jeyaprakash and Hoy 2000; Saridaki and Bourtzis 2010) that can be either primary for the host survival (Dedeine *et al.* 2003) or facultative. Facultative *Wolbachia* infections can either result in a fitness improvement (Dedeine *et al.* 2003) or can be detrimental (Min and Benzer 1997; Fry *et al.* 2004).

Within Tephritidae family, *Wolbachia* have been recorded as a facultative symbiont of several species of *Anastrepha* genus (Werren *et al.*, 1995; Selivon *et al.*, 2002) as well as of *Bactrocera dorsalis* (Sun *et al.*, 2007), *Rhagoletis pomonella* (O'Neill *et al.*, 1992), *Rhagoletis cerasi* (Riegler and Stauffer, 2002; Arthofer *et al.*, 2009), *Ceratitis capitata* (Rocha *et al.*, 2005) and *Dacus oscillatoria* (Kittayapong *et al.*, 2000). The presence of *Wolbachia* in fruit flies has begun of interest for the scientific community because this bacterium is well known to manipulate host reproduction. The bacteria inducing cytoplasmic incompatibility (CI), male killing, feminization or parthenogenesis (Stouthamer *et al.* 1990; Hoffmann and Turelli 1997; Rigaud 1997; Hurst *et al.* 1999) and thus, can be utilized in the development of the Incompatible Insect Technique (IIT). Some research groups have started trials to evaluate the effectiveness of this control strategy on *Ceratitis capitata* (Riegler and Stauffer 2002; Zabalou *et al.* 2004; 2009) and *B. oleae* (Apostolaki *et al.*, 2011) with remarkable results.

Thus, it is evident that the role of symbiosis between bacteria and fruit flies can deeply influence the impact of these pests on agriculture and that accurate investigations of these interactions may

lead to innovative and environmentally friendly strategies for the fruit flies control. Therefore, in the last ten years, several papers have been focused on the endosymbiosis studies in pests of economic interest and this topic is becoming a new frontier for the scientific community.

1.2.3 "*CA.* ERWINIA DACICOLA": THE PRIMARY ENDOSYMBIONT OF *B. OLEAE*

As we already mentioned, the first evidence of hereditary bacterial symbiosis in *B. oleae* dates back to the beginning of the twentieth century (Petri, 1909). The bacteria observed by Petri continuously multiply within the oesophageal bulb (Figure 3), developing masses that are then discharged into the midgut. The transmission to the offspring might be assured by the smearing of bacteria on the egg surface that the female act during oviposition through the contractile perianal glands that become filled with bacteria after they reach the sexual maturity. A bacterial cap-like mass is characteristically found nearby the egg's micropile and, when the embryo completes its development, the unhatched larva already contains the bacterial symbionts within its blind sacs. After the egg enclosure, the bacteria multiply inside intestinal caeca of all larval stages. Adult flies harbor large amounts of bacteria in the oesophageal bulb before emerging from their puparium (Petri, 1909). These early observations already supported the hypothesis that *B. oleae* bacterial endosymbionts are vertically transmitted from the mother to the offspring (Petri, 1909).

The same Author hypothesized that these symbiotic bacteria might belong to the *Pseudomonas savastanoi* (Smith) species, the agent of the olive knot disease. Later, the development of the DNA-based methodologies occurred in the last two decades, enabled the taxonomist to approach the description and the phylogenetic study of unculturable bacteria (Baumann and Moran, 1997). Thus in 2005, thanks to the molecular approach, Capuzzo *et al.* demonstrated that the *B. oleae* endosymbiont was not *P. savastanoi* and proposed a novel unculturable, inheritable taxon within the family Enterobacteriaceae, designed as "*Candidatus* Erwinia dacicola".

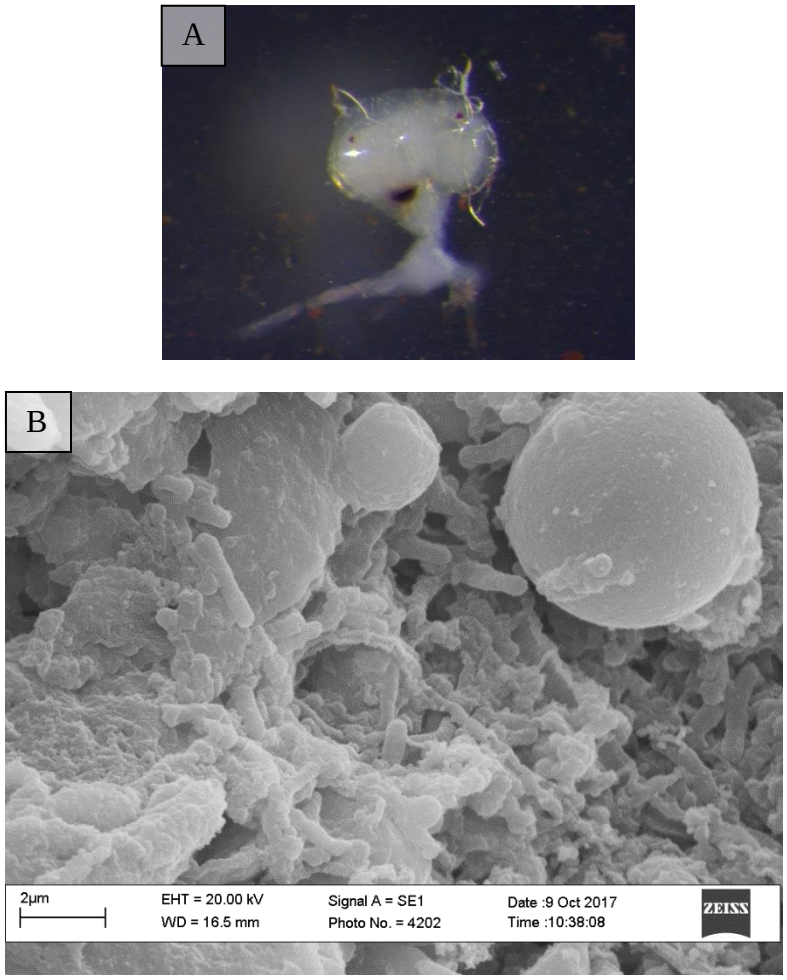

Figure 3: Picture of the oesophageal bulb under microscope after dissection (A) and picture of bacteria inhabiting the oesophageal bulb and of the biofilm that they produce with scanning electron microscope (B)

"Ca. Erwinia dacicola" belong to the 'Gammaproteobacteria' class with a cell envelope structurally coherent with the Gram-negative model. The similarity study of 16S rRNA gene sequence with BLAST algorithm against the GenBank database showed a 97% similarity with *Erwinia persicina* and *Erwinia rhapontici*, two plant pathogens belonging to the subgroup of *Erwinia amylovora*. This bacterial species is located exclusively in association with its host within larval blind sacs, imaginal oesophageal bulb, mid- and hindgut, anal glands, ovipositor and egg surface (Capuzzo *et al.*, 2005). The presence of the bacterial endosymbiont in the ovipositor, but not in the ovaries or testes, might confirm that "*Ca.* Erwinia dacicola" is vertically transmitted via smearing as the egg passes by the ovipositor diverticulum that is filled with bacteria (Sacchetti *et al.*, 2008). The mechanism throughout the symbiotic bacteria reach the embryo's blind sacs has not been clarified, but it can be hypothesized that the bacteria present on the egg surface pass through aeropilar or micropilar openings of the egg, in fact, Mazzini and Vita (1981) suggested it as a possible mechanism of symbiont transmission.

Otherwise, it can be hypothesized that the newly hatched larva ingests bacteria when it breaks the chorion (Sacchetti *et al.*, 2008).

In the study published by Capuzzo *et al.* (2005), all the PCR products of the bacterial 16S region that were sequenced were found to be identical confirming that a single bacterial species represent the entire or at least the most abundant fraction of the symbiotic microbiota of the olive fruit fly. The DNA was extracted from the oesophageal bulb, the mid-gut and the ovipositor of adult females of *B. oleae* belonging to wild Italian populations and all the samples showed the presence of "*Ca.* Erwinia dacicola" (Capuzzo *et al.*, 2005).

This study showed the first example of an obligate symbiotic relationship between a bacterium and a fruit fly. *B. oleae* also diverges from all the other species of the Tephritidae family for the characteristic structure of its oesophageal bulb and it could be hypothesized that these marked morphological differences may be explained by a coevolution with its specific symbiotic bacterial species (Capuzzo *et al.*, 2005).

Subsequent, recent studies have confirmed the presence of "*Ca.* Erwinia dacicola" in geographically distinct populations coming from Italy, Greece, Spain, Israel and South-Western USA (Belcari *et al.*, 2003; Capuzzo *et al.*, 2005; Sacchetti *et al.*, 2008; Silva *et al.*, 2008; Estes *et al.*, 2009. 2012a; Kounatidis *et al.*, 2008; Savio *et al.*, 2012. Ben-Yosef *et al.*, 2010. 2014). These data suggest that this endosymbiont has a specific and long-term association with its host.

"*Ca.* Erwinia dacicola" is commonly accompanied by other bacteria such as *Acetobacter tropicalis, Providencia* and *Pantoea* spp., which are considered transiently associated with the host gut and most likely acquired by the insect diet (Kounatidis *et al.*, 2008; Sacchetti *et al.*, 2008; Estes *et al.*, 2009; 2012a; Ben-Yosef *et al.*, 2015). The relative abundance of these bacteria seems to depend on "*Ca.* Erwinia dacicola" titer in insect gut, suggesting the presence of a regulation mechanism between these bacteria species (Estes, 2009).

During mass-rearing, the use of antibiotics in the artificial diet interrupts the natural transmission of the endosymbionts that are replaced by environmental bacteria. In these conditions, both adult and larval stages showed a remarkable fitness decrease (Estes *et al.*, 2012; Ben-Yosef *et al.*, 2015; Ras *et al.*, 2017).

The images acquired by transmission electron microscope (TEM) showed that the symbiotic bacteria are located intracellularly in all larval stages, within the cellular membrane of epithelial cells of the digestive tissue. On the contrary, in the adult stage, "*Ca.* Erwinia dacicola" resides

extracellularly and forms a bacterial biofilm into the digestive system lumen as in the oesophageal bulb, outside the cellular membrane of host cells (Estes *et al.*, 2009) (Fig. 3-B).

During the olive fly development, the bacterial endosymbiont switches from an intracellular existence in the larval stages to an extracellular existence in adults. This transition, that is very rare and of which there are only a few other examples reported in the literature (Cheng and Aksoy, 1999; Pais *et al.*, 2008), could be crucial to the bacteria survival in their holometabolous, polyphagous host. Nonetheless, "*Ca.* Erwinia dacicola" is the first case of an intracellular endosymbiont within tephritids (Estes *et al.*, 2009; Lauzon, 2003).

During metamorphosis, Diptera larvae degrade their digestive tissues that are then reassembled from the larval mid-gut regenerative cells in adult stage (Greenberg, 1959; Jiang *et al.*, 1997). Therefore, permanent bacterial endosymbionts must carry out a mechanism to survive during the degradation of larval tissues and recolonize the adult's ones. It could be hypothesized that the bacteria inhabiting the larval regenerative cells reestablish themselves in the adult gut (Estes *et al.*, 2009).

The bacterial amount of recently eclosed adults is very low, while the oesophageal bulb of older adults is filled with bacteria, this suggests a rapid multiplication of bacteria in the first days post-eclosion (Estes *et al.*, 2009). As soon as biofilms were established, thus after about 5-days post-eclosion, the endosymbionts cannot be removed from their host by oral administration of antibiotics (Estes *et al.*, 2009).

"*Ca.* Erwinia dacicola" is found to be associated with *B. oleae* in high frequency. Its presence in all life stages of different widespread populations, its vertical transmission to offspring and its skill to be located within the larval host cells imply that this bacterial species has a highly specific and long-term association with the olive fruit fly (Estes *et al.*, 2009; Paracer and Ahmadjian, 2000; Ishikawa, 2003).

1.2.4 *B. OLEAE* ADULT FITNESS ADVANTAGES DUE TO PRESENCE OF ITS SPECIFIC ENDOSYMBIONT "*CA.* ERWINA DACICOLA"

Nitrogen is the most common limiting factor in insect nutrition since proteins synthesis depends on the availability of dietary amino acids of which the essential ones cannot be synthesized *de novo* (Dadd, 1985). The association between insects and bacteria has been often evolved to overcome nutritionally unbalanced diets constraints. Monophagous insects feeding on a nitrogen-poor substrate are particularly liable to such restrictions (Douglas, 2006; 2013) but also polyphagous insects can difficulty find an optimal and predictable nutrient input (Kaufman *et al.*, 2000). In fact, both kinds of insects are known to frequently satisfy their protein needs throughout the association with bacterial symbionts, which enable them to synthesize essential amino acids, fix nitrogen or recycle nitrogenous waste compounds (Zientz *et al.*, 2004; Douglas, 2009. 2013; Dillon and Dillon, 2004; Engel and Moran, 2013).

The olive fruit fly has a monophagous feeding strategy in the larval stages that exclusively feed on the olive fruit mesocarp while the adult stage is polyphagous and exploits various substrates of which plant exudates, homopteran honeydews, and bird droppings are considered the primary resources providing carbohydrates and nitrogen (Bateman, 1972; Fletcher, 1987. Drew and Yuval, 2000). There is evidence that these substrates considerably endorse the insect fitness compared to a diet consisting exclusively of carbohydrates (Tsiropoulos, 1977).

Nonetheless, these food sources are mostly rich in carbohydrates but moderately poor in amino acids (Wackers, 2005; Lundgren 2009) and often the amino acids content is mainly composed of non-essential ones (Wackers, 2005; Douglas 2006). On the other hand, bird drops are rich in uric acid, urea and ammonia salts (Davis, 1927; Tsahar *et al.*, 2005) but insects are mostly incapable to degrade purines and urea and these nitrogen compounds cannot be exploited for their nutrition (Cochran, 1985). Thus, the actual nutritional value of the adult olive flies' diet may be quite restrictive and unbalanced in its amino acid composition. Furthermore, the protein content in diet is particularly important for adult females, which require a considerable amount of essential amino acids to achieve a successful maturation of their eggs (Tsiropoulos, 1980; 1983; 1984) and this reproductive demand seems to be unlikely satisfied by their poor and unbalanced diet (Tsiropoulos 1977; Drew and Yuval 2000).

Since nutrient acquisition for adult olive flies can be spatially and temporally patchy, the formation of a symbiotic bacterial biofilm in the digestive lumen may provide resistance to low nutrients periods and other stresses (Davey and O'Toole, 2000; Estes *et al.*, 2009).

It has also been hypothesized that symbiotic bacteria housed in the oesophageal bulb and subsequently forming biomasses discharged into the gut, might be digested by their host, thus providing a direct nitrogen source for adult olive fly, as described for other fruit flies (Estes *et al.*, 2009; Drew and Yuval 2000; Drew and Lloyd 1991; Lemos and Terra 1991).

Ben-Yosef *et al.* (2010) have recently pointed out the role of the gut microbiota, mainly composed of "*Ca.* Erwinia dacicola", on the adult olive flies' nutrition and fertility. *B. oleae* adult females treated with antibiotics (aposymbiotic specimens) and fed on diets provided with non-essential amino acids have shown a significantly lower fecundity in comparison with the non-treated counterparts (symbiotic specimens) fed on the same diet. The number of eggs produced by the aposymbiotic females was comparable with the one produced by symbiotic females fed on sugar diets without any nitrogen compounds addiction, revealing that *B. oleae* is unable to exploit non-essential amino acids without the presence of its specific endosymbiont and suggesting that these bacteria exploit the nitrogen available in the host diet to complement the missing amino acids required for protein synthesis. (Ben-Yosef *et al.*, 2010; 2014).

Similar results have been obtained in another experiment (Ben-Yosef *et al.*, 2014) in which symbiotic and aposymbiotic females were fed on diet provided either with urea or with bird droppings, which contain a variety of waste nitrogenous compounds including uric acid, urea and ammonia (Davis, 1927; van Tets *et al.*, 2001; Tsahar *et al.*, 2005). The aposymbiotic females produced a significantly lower amount of eggs in comparison with their symbiotic counterparts, accounting for more than a two-fold decrease in fecundity, and responded as if completely deprived of nitrogen. These results reveal that the presence of the gut endosymbionts is essential for the olive fly to exploit the nitrogenous waste metabolites present in its natural diet as a nitrogen source and suggest that these bacteria are involved in the metabolism of uric acid, urea, and ammonia (Ben-Yosef *et al.*, 2014). It can be hypothesized the activity of a bacterially derived urease, into the insect gut, that might hydrolyze urea, generating ammonia that is the initial precursor of *de novo* amino acid synthesis. Thus, we can assume that ammonia might be successively used by bacteria for synthesizing amino acids and that the host females satisfy their protein needs by utilizing these bacterially derived amino acids (Ben-Yosef *et al.*, 2014).

On the contrary, the aposymbiotic and symbiotic females showed a similar amount of produced eggs either when they were fed only on sucrose diet or on diets provided with a proper quantity of essential amino acids. These results indicate that "*Ca.* Erwinia dacicola" plays an essential role in the olive fly's nutrition and fertility only when there is a nitrogen source in the host diets

and when this source is composed of non-essential amino acids and/or nitrogenous waste metabolites, that are, anyway, the most common nutritional circumstances occurring in the field environment (Ben-Yosef *et al.*, 2010; 2014).

Consequently, we can assume that "*Ca.* Erwinia dacicola" is able to use either the non-essential amino acids or the nitrogenous waste metabolites present in the fly's diet as building blocks for essential amino acid synthesis, which can successively be utilized by its host in their free form or integrated into a bacterial protein after their secretion into the gut. Thus, this endosymbiont promotes the olive fly females nutrition and oogenesis by compensating for essential amino acid deficiencies in its host diet (Ben-Yosef *et al.*, 2010; 2014).

Earlier studies of bacteria isolated from the gut of *B. oleae* and other fruit flies (Hellmuth, 1956; Lauzon *et al.*, 2000; Robacker and Lauzon, 2002) displayed that some of these bacteria were able to utilize uric acid and urea and to recycle these compounds into the host gut. These biosynthetic pathways seem now to be present within a heritable, obligate symbiont such as "*Ca.* Erwinia dacicola", suggesting the importance of this symbiosis to the natural history and the evolution of its host (Ben-Yosef *et al.*, 2014).

1.2.5 *B. OLEAE* LARVAL FITNESS ADVANTAGES DUE TO PRESENCE OF ITS SPECIFIC ENDOSYMBIONT "*CA.* ERWINA DACICOLA"

Another peculiarity of the olive fruit fly is the feeding strategy of its larval stages. While most fruit flies lay their eggs in ripe fruit (Fletcher, 1987), which better support the larval development (Greany, 1989; Messina and Jones, 1990; Joachim-Bravo *et al.*, 2001; Rattanapun *et al.*, 2009), *B. oleae* larvae predominantly develop in unripe olive fruit, permitting them to complete several generation before the fruit ripening (Kapatos and Fletcher, 1984; Neuenschwander, 1985).

Unripe fruits are generally resistant to herbivores and pathogens attack thanks to the high content of secondary metabolites with antimicrobial, anti-nutritive and toxic effects (Whitehead *et al.*, 2013; Gutierrez-Rosales *et al.*, 2012). The unripe fruit of *Olea europaea* is no exception; in fact, it contains several secondary compounds, the most abundant of which is oleuropein, a bitter phenolic glycoside that can contribute up to 14% of the fruit's dry weight (Alagna *et al.*, 2012; Amiot *et al.*, 1986; Gutierrez-Rosales *et al.*, 2012). Phenolic compounds are well known to act as strong protein alkylators once activated that prevent herbivores and pathogens attack (Bennett and Wallsgrove, 1994; Felton and Gatehouse, 1996; Taiz and Zeiger, 2010; Pentzold *et al.*,

2014). After enzymatic activation by plant β-glucosidase and phenoloxidase, oleuropein forms highly reactive aldehydes and quinones, which are able to cross-link proteins contained in plant tissues forming high molecular weight aggregates throughout a covalent binding of their lysine that is an essential nutrient (Konno *et al.*, 1999; Koudounas *et al.*, 2015). Activated oleuropein has also a strong antimicrobial effect inhibiting bacteria and fungi attack (Brenes *et al.*, 2011; Dobler *et al.*, 2011).

It has been demonstrated that insects feeding on *Ligustrum* spp., a plant genus belonging to the Oleaceae family that also contains a high amount of oleuropein, undergo to growth arrestment caused by decreased lysine content in their diet (Konno *et al.*, 2009; 2010). Therefore, it has been hypothesized that a similar defense mechanism, characterized by lysine amino acid unavailability with a consequent lack of protein content, is present in the unripe olive fruits (Spadafora *et al.*, 2008; Kubo *et al.*, 1985; Koudounas *et al.*, 2015).

A high larval mortality has been described in laboratory condition, adding antibiotics to the adult diet (Fytizas and Tzanakakis, 1966) but also in field trials where a remarkable negative effect of copper treatments, that could act as symbioticides, have been reported on larval growth (Tzanakakis in 1985; Belcari and Bobbio 1999; Belcari *et al.* 2005). These results support the hypothesis that the presence of endosymbiotic bacteria in the blind sacs of the larvae midgut might positively influence the larval fitness and development.

In 2015, Ben-Yosef *et al.*, published a study in which the interaction between olive fly larvae, their symbiotic bacteria, and olive fruit chemistry have been investigated. Hypothesizing that the phenolic compounds present into the unripe olive fruits might carry out a substantial restriction on the larval nutrition by reducing the nutritional value of protein, the possibility that this constraint could be overcome by their symbiotic bacteria have been evaluated (Ben-Yosef *et al.*, 2015).

Symbiotic and aposymbiotic larval development either into ripe or unripe olive fruits had been monitored. While symbiotic larvae were able to develop in unripe olive fruits, their aposymbiotic counterparts could not reach the pupal stage demonstrating that "*Ca.* Erwinia dacicola" is essential for the development of *B. oleae* larvae into unripe olive fruits. On the contrary, both symbiotic and aposymbiotic larvae successfully developed in ripe olive fruits, nonetheless, the aposymbiotic larvae completed their development nearly 2 days later and their weight was approximately 12% less than their symbiotic counterparts. These results suggest that even if the presence of this specific endosymbiont is not essential for *B. oleae* larvae to develop into ripe

fruit, it significantly accelerate their development, most likely increasing larval nutrition (Ben-Yosef *et al.*, 2015).

The pattern observed in larval development suggest that the more the lysine is lost and protein complexes accumulate following incubation of ovalbumin in fruit extract, the more the larvae depend on the presence of its symbiont to successfully develop. In fact, unripe olive fruits extract showed a greater capacity to bind ovalbumin together and destroy lysine residues than ripe olive fruits one, indicating that probably oleuropein is more active in unripe olive fruits (Ben-Yosef *et al.*, 2015). Furthermore, the content of oleuropein and β-glucosidase—the main activating enzyme of oleuropein— progressively decrease during ripening, reaching a low level in ripe fruit (Briante *et al.*, 2002; Alagna *et al.*, 2012; Amiot *et al.*, 1986), while the content of lysine of olive pulp proteins substantially increase (Zamora *et al.*, 2001).

Thus, the phenology of oleuropein during ripening seems to be the main process influencing lysine and protein content in the olive fruit pulp available for the nutrition of *B. oleae* larvae and it is probably the key reason for the ripening-dependent capability of aposymbiotic larvae to develop into the olive fruit (Ben-Yosef *et al.*, 2015).

Ben-Yosef *et al.* (2015) suggested that oleuropein is not toxic but has an anti-nutrient effect (Felton and Gatehouse, 1996; Konno *et al.*, 1999), causing a severe nutritional restriction by lysine deficiency in on olive fly larvae which lacks their specific endosymbiont (Tsiropoulos, 1984). Furthermore, oleuropein might act as enzymes activity inhibitor, decrease the digestibility of dietary protein (Kroll *et al.*, 2003; Felton and Gatehouse, 1996; Pentzold *et al.*, 2014) and prevent any premature decomposition of the olive pulp due to bacterial or fungal proliferation, which may enhance larval nutrition (Brenes *et al.*, 2011). Thus, it has been assumed that olive fly larvae feeding on unripe fruits have to deal with high molecular weight and cross-linked protein aggregates, with a low lysine content and most likely hard to digest and that these constraints are counteracted by their bacterial symbionts (Ben-Yosef *et al.*, 2015).

Although the precise mechanism by which "*Ca.* Erwinia dacicola" improve the larval development is still unknown, some hypotheses have been proposed. These bacteria undoubtedly provide a source of protein or amino acids to the larvae during their development (Ben-Yosef *et al.*, 2015). This might be realized throughout the neutralization of plant defense compounds as has been reported for other symbiotic bacteria associated with fruit flies (Boush and Matsumura, 1967; Lauzon *et al.*, 2003). Furthermore, they may act to dissociate the oleuropein-protein

complexes secreting polyphenol-degrading enzymes or polyphenol-binding polymers (e.g. Smith *et al.*, 2005; Dowd, 1992).

"*Ca.* Erwinia dacicola" is phylogenetically close to some necrotrophic free-living *Erwinia* species, which are well known to exploit living plant tissue as a nutritional source throughout the secretion of extracellular enzymes (Mazzon *et al.*, 2008; Barras *et al.*, 1994). This phylogenetic proximity supports the hypothesis that this endosymbiont is somehow involved in the secretion of degrading enzymes targeting on plant secondary compounds (Ben-Yosef *et al.*, 2015).

It is also possible that, in addition to their detoxification activity, these bacteria were utilized by their host as a direct and renewable source of balanced protein and amino acids, by their digestion into the larvae gut, thus enhancing the larval nutrition (Ben-Yosef *et al.*, 2015). This hypothesis is supported by the knowledge about the evolution of Tephritidae, which probably have saprophagous ancestors feeding on microbe-rich, rotting plant tissues (Diaz-Fleischer *et al.*, 2000). Furthermore, fruit flies are also well known to have deep physiological adaptations for lysing and digesting bacteria (Lemos and Terra, 1991; Terra and Ferreira, 2012). Moreover, *B. oleae* midgut caeca has a contractile nature and the bacteria are often discharged into the larval gut during development (Petri, 1909) thus, suggesting that endosymbiotic bacteria can be eventually digested (Ben-Yosef *et al.*, 2015).

A transcriptomic and proteomic approach has been recently performed to investigate the interaction between *B. oleae*, "*Ca.* Erwinia dacicola" and the olive fruit (Pavlidi *et al.*, 2017). The gene expression of wild olive fly larvae during their development in artificial diet and unripe and ripe olive fruit as well as the endosymbiont gene expression, has been analyzed in order to elucidate the mechanism that allow the *B. oleae* larval stages to overcome the host plant defenses and in particular to deal with the presence of high concentration of oleuropein in unripe fruits (Pavlidi *et al.*, 2017).

Olive fruit fly larvae developing in unripe fruit showed a remarkable overexpression of genes encoding detoxification enzymes. Under the same conditions, the larvae endosymbionts presented an overexpression of genes involved in the secretion of an inhibitor of oleuropein compound. It is still not clear if the secretion of degrading enzymes occurs in the insect or in their bacterial endosymbiont but it can be hypothesized that both partners of this symbiosis interaction contribute to oleuropein degradation (Pavlidi *et al.*, 2017).

2. AIM OF THE THESIS

While some investigations on the role that endosymbiotic bacteria play in the olive fly fitness have been recently pinpointed, little is known about the possibility of utilizing symbioticides compounds that may interfere with the "*Ca.* Erwinia dacicola" / Olive Fly interaction reducing the destructiveness of this widespread pest.

Taking into account the fact that "*Ca.* Erwinia dacicola" is essential for the larval survival in unripe olive fruits and remembering that the first 4 or 5 generations develop in unripe olive fruits, it is evident that use of efficient symbioticides could represent a substantial step forward in *B. oleae* pest management.

Therefore, the identification of environmentally friendly compounds that could be used as symbiosis inhibitors seems to be an interesting but poorly explored field of research that could lead to the development of new solutions/options to enhance the olive fruit fly integrated pest management.

The aim of the research project here-presented is based on this approach. Promising microbial metabolites and Copper Oxychloride solution were selected for this purpose and these substances were administered to wild specimens of *B. oleae* during the experimental phase. Assessment of biological parameters (mortality rate, the amount of ingested diet, females' capability to mate and lay eggs, and newly hatched larvae ability to develop in unripe olive fruits and cause damage) was carried out for each treatment.

The research project was designed to avoid the influence of the symbionts presence on the nutrition of the adult stages. In fact, the selected substances were administered within two different diets respectively consisting of sucrose only (S-diet) and of sucrose and yeast hydrolysate mixture ((S+P)-diet) which contained all the essential amino acids required by the adult's development and reproduction. As reported by Ben-Yosef (2014), in these two opposite nutritional conditions the olive fruit fly cannot benefit from its specific endosymbiont since, in the case of the S-diet, the actual lack of nitrogen sources make impossible for the symbionts to synthesized the essential amino acids to complement the flies nutritional needs, and, in the case of the (S+P)-diet, the presence of all the elements required by the fly's development make useless the presence of the symbionts.

Data on differential adult fitness (longevity, ability to mate and lay eggs) were utilized to identify treatments whose effects are probably linked to a toxic effect of the substances rather

than to the symbiont decrease. On the contrary, substances were considered efficient symbioticides if a strong effect on the larval survival was recorded (without significant influence on adult fitness).

Moreover, for further evidence that the larval fitness decrease was due to the symbiotic titer reduction, molecular analysis by real-time PCR was performed on the mid-gut and the oesophageal bulb of the treated flies, in order to quantify the fly's beneficial bacterial population corresponding to each treatment. In any event, broadening the knowledge about the symbiosis between *B. oleae* and its specific endosymbiont and better understanding the mechanisms of microbiome alteration and its consequence on the insect fitness may provide valuable insight into control options of this significant agricultural pest.

3. MATERIALS AND METHODS

3.1 FITNESS TESTS

3.1.1 SAMPLING, ORIGIN, AND MAINTENANCE OF *B. OLEAE* WILD POPULATION

Experiments were conducted with wild flies emerged from field infested olive fruits of the same cultivar to achieve a physiologically uniform population. Olive fruits were weekly collected from olive trees located at the Department of Agricultural Sciences of the University of Naples, Federico II (Portici, NA), from September to December of 2015 and 2016. Trees are cultivated for scientific purposes and have never undergone to chemical treatment.

At the beginning of June 2015 and 2016. some branches of selected trees were enveloped in 100L mesh fabric to avoid the pest attack and provide uninfested olive fruits, required during the research project. The rest of the branches were left unenveloped to permit the pest attack and provide the field infested olive fruits.

2 kg of infested olive fruits were weekly collected, placed in plastic trays over a paper layer and incubated. Mature larvae and pupae were daily collected and placed in 100L mesh cages. After emergence, adults were separated by sex, divided into groups of 25 specimens and placed in cylindrical cages of a diameter of 20 cm and a height of 25cm.

All experiments were conducted in a controlled environment (24 ± 2°C, 60 ± 10% RH and 12/12 LD cycle).

3.1.2 TEST OF FITNESS REDUCTION THROUGH MICROBIOME ALTERATION

In order to verify if the alteration the *B. oleae* microbiome – and in particular the reduction of the endosymbiont presence into the fly's digestive system – could reduce the adult and larval fitness, a selection of microbial metabolites and copper oxychloride were orally administered to the new hatching adults for a 20 days' time span.

The compounds to be orally administered were selected for their well-known antibacterial properties (Sivasithamparam and Ghisalberti, 1998; Keswani *et al.*, 2014; Howell, 2003; Vinale *et al.*, 2006; 2014; Dias *et al.*, 2012; Chiang *et al.*, 2009; Mukherjee *et al.*, 2006; Pascale *et al.*,

2017 Asaka and Shoda 1996; Chen and Wu 1999; Harris and Adkins 1999; Ferreira *et al.* 1991; Sholberg *et al.* 1995; Mari *et al.* 1996; Raaijmakers *et al.* 2002; He *et al.* 1994). Microbial metabolites were provided by the research team of the IPSP-CNR of Portici (NA) that extracted them from different *Trichoderma* species and *Bacillus subtilis* (Table 2).

Copper oxychloride has been hypothesized to act as symbioticides in the olive fruit fly (Rosi *et al.*, 2007; Sacchetti *et al.*, 2004; Belcari and Bobbio, 1999; Tzanakakis, 1985), for this reason, it was introduced into this research project to effectively test its antibacterial effect on the *B. oleae* microbiome.

Table 2: Microbial metabolites list with relative origin organism and lower concentration with antibacterial properties

Microbial metabolites	Organism	Antibacterial properties concentration
Harzianic acid	Trichoderma harzianum	10^{-2} M
6-pentyl-α-pyron	Trichoderma atroviride	10^{-3} M
Viridiol	Trichoderma virens	10^{-4} M
Lipopeptides mixture	Bacillus subtilis	100 ppm

The selected compounds were directly added to the fly's diet in two or three 5-fold serial dilutions. Two different adult diets were used, respectively consisting of sucrose (S-diet) and of 1 : 1 (w/w) mixture sucrose and yeast hydrolysate ((S+P)-diet), to compare the effect of the treatments depending on the nutritional state of the flies. S-diet, which is deprived of any source of proteins, represent a nutritionally unbalanced diets constraint while (S+P)-diet is provided with essential and non-essential amino acids and is thus considered nutritionally complete.

As a negative control, cohorts of females and males were separately fed on the same diets with the addition of antibiotics (Piperacillin, Sigma, 100 µg/mL). It has been demonstrated that this treatment effectively clears the endosymbiotic bacteria from the fly's digestive system (Ben-Yosef *et al.* 2008; 2010; 2014). Simultaneously, reciprocal adult groups fed with diets with no added substances were utilized as a positive control.

A number of 28 different treatment was tested (Table 3), each with 3-5 biological repetition. Every single repetition was carried out on 50 specimens, 25 females and 25 males, separately for the first 14^{th} days and together for the subsequent 6 days.

Table 3: List of the treatments with relative substances, concentrations and diet type.

Treatment	Added substance and concentration	Diet
S CONTROL	No substances added	S
S+P CONTROL	No substances added	S+P
S ANTIBIOTICS	Piperacillin 0.01‰	S
S+P ANTIBIOTICS	Piperacillin 0.01‰	S+P
S COPPER OX. 0.5%	Copper oxychloride 0.5%	S
S+P COPPER OX. 0.5%	Copper oxychloride 0.5%	S+P
S COPPER OX. 0.1%	Copper oxychloride 0.1%	S
S+P COPPER OX. 0.1%	Copper oxychloride 0.1%	S+P
S COPPER OX. 0.02%	Copper oxychloride 0.02%	S
S+P COPPER OX. 0.02%	Copper oxychloride 0.02%	S+P
S HARZIANIC ACID 0.5%	Harzianic acid 0.5%	S
S+P HARZIANIC ACID 0.5%	Harzianic acid 0.5%	S+P
S HARZIANIC ACID 0.1%	Harzianic acid 0.1%	S
S+P HARZIANIC ACID 0.1%	Harzianic acid 0.1%	S+P
S+P HARZIANIC ACID 0.05%	Harzianic acid 0.05%	S+P
S VIRIDIOL 0.5%	Viridiol 0.5%	S
S+P VIRIDIOL 0.5%	Viridiol 0.5%	S+P
S VIRIDIOL 0.1%	Viridiol 0.1%	S
S+P VIRIDIOL 0.1%	Viridiol 0.1%	S+P
S+P VIRIDIOL 0.05%	Viridiol 0.05%	S+P
S 6PP 5%	6-pentyl-α-pyron 5%	S
S+P 6PP 5%	6-pentyl-α-pyron 5%	S+P
S 6PP 1%	6-pentyl-α-pyron 1%	S
S+P 6PP 1%	6-pentyl-α-pyron 1%	S+P
S BACILLUS 5%	Lipopeptides mixture 5%	S
S+P BACILLUS 5%	Lipopeptides mixture 5%	S+P
S BACILLUS 1%	Lipopeptides mixture 1%	S
S+P BACILLUS 1%	Lipopeptides mixture 1%	S+P

3.1.3 ADULT AND LARVAL FITNESS TESTS

Each group of 25 specimens of the same sex was maintained for 14 days after emergence, which is a time required by females to mature sexually (Zervas, 1982), in a cage supplied with water and one of the different diets with the addition of the above-mentioned compounds depending on the experiment.

The exact weight of the diet supplied in each cage was recorded on the day 1st and successively on the day 14th to determine the mean amount of treatment administered to each specimen. The number of dead specimens was daily recorded from the emergence until the end of the experiments.

On their 14th day, females were joined with the males to permit the mating and simultaneously 25 unripe and un-infested olive fruits were supplied into the cages (1 olive fruit/female) to provide a natural substrate for female's oviposition. The supplied olive fruits were collected from the enveloped branches and carefully examined to avoid the use of already infested fruits.

After 3 days, the olive fruits were collected and replaced with the same amount of a new group, which was successively collected after 3 days. These two groups of olives, corresponding to the first two oviposition tests, were separately processed, to verify if the time span required for oviposition varied among the different treatments.

Half of each olive group was immediately dissected under microscope and the number of eggs laid was recorded. The remaining half was incubated for 2-4 weeks to record the differential rate of offspring adult emergence among the treatments.

The number of eggs/female, after 14 days of treatment, was used to assess the fitness of adults. Since the number of females was equal to the number of un-infested olive fruits exposed into the cage, it was assumed that the number of laid eggs in each olive fruit corresponded to the number of laid eggs for female. Olive fruits were exposed twice for a period of 3+3 days, and the number of laid eggs in the first exposition period was recorded separately from the second one.

The number of laid eggs was recorded separately for the two oviposition tests, the first corresponding to the day 14th-17th of treatment, immediately after that males and females were placed into the same cage, and the second corresponding to the day 17th-20th. Thus, analyzing the differences between the first and the second oviposition test in each group, it was possible to verify the time required by flies to mate and by females to mature eggs, associated with the

different administered treatment. An addition of the average number of laid eggs in first and second oviposition test was also calculated to have an index of the total females' fertility regardless of the time span.

The larval fitness was tested on the F1 larvae, offspring of the treated females derived from the eggs deposited during the tests. As larval fitness index, the number of eggs that completed the larval development and survived until the adult stage was used. The average number of F1 offspring per female emerging from the olive fruits exposed to the treated females was analyzed to investigate the efficiency of the treatments as symbioticides. The hypothesis was that the lower amount of the offspring was due to the lack of the specific endosymbiont into the larvae ecdysed from the eggs laid by the treated females. The number of the F1 adults was reported separately for the two oviposition test, the first corresponding to the day 14th-17th of treatment, immediately after that males and females were placed into the same cage, and the second corresponding to the day 17th-20th. An addition of the average number of offspring in first and second oviposition test was also calculated to have an index of the total larval fitness regardless of the time span.

Comparing the number of laid eggs and the number of offspring per female the larval mortality rate was calculated for each treatment.

At the end of the second oviposition test, all the treated adults were frozen (-80°C) and stored until further processing.

3.2 MOLECULAR ANALYSIS OF MICROBIOME ALTERATION

3.2.1 ENDOSYMBIOTIC BACTERIA QUANTIFICATION BY REAL-TIME PCR

In order to understand whether the differences between the fitness of the treated flies and the fitness of the control groups truly depended on the antimicrobial activities of the treatment instead of a hypothetical toxic effect, the bacterial titer of the flies after 20 days of treatment was evaluated.

The endosymbiotic bacteria quantification was carried out by real-time PCR on 12 females of each treatment group, divided into 3 pools of 4 specimens.

Real-time PCR was used to quantify the number of copies of "*Ca.* Erwinia dacicola" 16S rDNA present in each sample of treated files in comparison with the positive control (no treatment) and the negative control (treated with antibiotics). Real-time PCR was carried out on biological triplicates and technical triplicates for each treated group

3.2.2 DNA EXTRACTION

The frozen flies were surface-sterilized by vortexing for 15 s in a 1% sodium hypochlorite –0.1% Triton X solution, rinsed twice with distilled water and then dissected under sterile conditions in a laminar flow hood to separate the head and the abdomen where the oesophageal bulb and mid-gut are located, respectively.

After dissection, the pool of 4 heads and the pool of 4 abdomens were separately used for the genomic DNA extraction. The DNA was extracted using the PureLink® Genomic DNA Mini Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

At the end of the extraction, 2 DNA samples of the same 4 flies were obtained, one from the heads and one from the abdomens. These 2 kinds of DNA were always processed separately. The reason why the DNA was extracted separately from these 2 body parts was to investigate the changes of the bacterial titer and simultaneously the microbiome composition within the two organs: the oesophageal bulb and the mid-gut. Taking into account that the oesophageal bulb is a specific diverticulum coevolved with the endosymbiont "*Ca.* Erwinia dacicola" while the mid-gut is generally inhabited by several bacterial species, it was considered interesting to study the different patterns of bacterial titer and microbiome alteration between these 2 body parts.

The extracted DNA quality and concentration were evaluated using the Varioskan (Thermo Fisher Scientific). All the DNA samples were stored at -80°C until further processing.

3.2.3 PRIMERS, HOUSEKEEPING GENE, AND REAL-TIME PCR CONDITIONS

The amplification of "*Ca.* Erwinia dacicola" 16S rDNA region was obtained with primers EdEnRev (Estes *et al.* 2012) and EdF1 (Estes *et al.* 2009), which generate a 90pb amplicon. These primers were already tested in a study on the relative abundance of "*Ca.* Erwinia dacicola" across life stages of B. oleae (Estes *et al.* 2012).

To normalize data a housekeeping gene of the *B. oleae* genomic DNA was used: β-actin gene. Housekeeping genes are expressed at consistent and stable level across different tissues and under mostly all kinds of experimental conditions. The products of these genes are typically enzymes or proteins needed for the cell survival and maintenance and are extremely abundant in all cell types; this is the case of actin, tubulin, and ribosomal RNA. Hence, these genes are used as a reference to normalize real-time PCR data.

The amplification of a portion of the β-actin gene was obtained with a couple of primers designed with Primer Express Software (Thermo Fisher Scientific). 3 couples of primers were designed and their efficiency was tested. The most efficient couple, Act2F (5'-GCAGAGCAAACGTGGTAT-3'), and Act2R (5'-TGTGATGCCACACTTTCT-3'), which generates a 91pb amplicon, was chosen.

Real-time PCR experiments were carried out with SYBR Green PCR Master Mix (Thermo Fisher Scientific) in 13µl of total volume, containing 3µl of diluted genomic DNA, 6.5µl of Master Mix and a solution of primers with a final concentration of 300 nM. The cycle used by Estes (2012) was tested and adapted. The experiments were performed with a Step-one Cycler (Life Technologies) as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. Reactions were followed by a quality control melting curve and terminated by a cooling.

Negative controls and 2 standard curves, one for "*Ca.* Erwinia dacicola" 16S rDNA and another one for *B. oleae* β-actin gene, were run on each 96 wells plate.

3.2.4 STANDARD CURVES

Standard curves were generated using purified amplicons, produced with the above-mentioned primers, as the template. A classic PCR was performed with DreamTaq PCR Master Mix (Thermo Fisher Scientific) in a total volume of 50µl, using 3µl of genomic DNA extracted from the control samples and 2µl 10mM of primers EdEnRev (Estes *et al.* 2012) and EdF1 (Estes *et al.* 2009), for the amplification of "*Ca.* Erwinia dacicola" 16S rDNA, and Act2F and Act2R for the amplification of *B. oleae* β-actin gene.

The PCR products were undergone by agarose gel electrophoresis and the bands were successively extracted from the gel and purified with QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions. The exact concentration of purified amplicons was determined using the Qubit 2.0 Fluorometer (Life Technologies).

The following equation was used to calculate the numbers of amplicon copies into the purified template with a predetermined concentration: weight of PCR fragment (g/µl) / (660 g/mol × pair bases number of the PCR fragment) × (6.023 × 10(23)) = number of genomic copies per microliter (Malorny *et al.*, 2003).

Knowing the number of copies of the templates per microliter, it was possible to make five 10-fold serial dilutions with a certain number of copies, starting from the most concentrate with 1.00E+07=10000000 number of copies until the less concentrate with 1.00E+03=1000 number of copies. These serial dilutions were used to generate the standard curves.

Standard curves were performed with 3 different concentration of primer: 300mM, 600mM, and 900mM. In order to verify the concentration with the highest efficiency and lowest amount of aspecific amplification products, melting curves and standard curves equation were analyzed. Best results were obtained with the 300mM concentration of primers in both standard curves.

The equation of "*Ca.* Erwinia dacicola" 16S rDNA standard curve was: $y = -3.3213x + 34.689$ with an efficiency of 100.02%, and an $R^2 = 0.9939$. The equation of B. oleae β-actin gene standard curve was: $y = -3.3290x + 34.531$with an efficiency of 101.34%, and an $R^2 = 0.9999$. Both curves had very similar efficiency and slope allowing the use of $2^{-(\Delta\Delta Ct)}$ method for the relative quantification.

3.2.5 BACTERIAL QUANTIFICATION

The abundance of "*Ca.* Erwinia dacicola" 16S rDNA was calculated relative to the *B. oleae* β-actin gene reference using the $2^{-(\Delta\Delta Ct)}$ method. The ΔCt between the reference gene (*B. oleae* β-actin) and the target gene (*Ca.* Erwinia dacicola" 16S rDNA) was calculated for each sample using the following equation: $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene.}}$ Then, the ΔΔCt between the ΔCt of the treated samples and the average ΔCt of the control groups was calculated using the following equation: $\Delta\Delta Ct = \Delta Ct_{\text{ treatment}} - \text{mean } \Delta Ct_{\text{ control}}$. Finally, the fold change $2^{-(\Delta\Delta Ct)}$ was calculated and averaged.

Then the data obtained were log-transformed and the logarithm of the fold change $2^{-(\Delta\Delta Ct)}$ was used as an index of the relative abundance of the bacterial titer in comparison with the control.

3.3 STATISTICAL ANALYSIS OF RESULTS

Statistical analyses of the results were carried out with Minitab 18 Statistical Software ®, PAST 3 ® and Microsoft Excel ®.

3.3.1 FITNESS DATA

About the fitness tests data, the distributions were analyzed and the summary statistic calculated. The normal distribution of each parameter dataset was verified with a Shapiro-Wilk test with a $W \geq 0.05$ and a p normal ≥ 0.05. The dataset was divided into 4 subsets by sex and diet type: ♀ - S-diet; ♂ - S-diet; ♀ - (S+P)-diet; ♂ - (S+P)-diet for mortality and amount of ingested diet parameters. The dataset of the number of laid eggs per female and the number of offspring per female were divided into 6 subsets: 2 subsets for the total number of laid eggs divided by diet type, and 4 subsets divided by oviposition test and diet type: S-diet - I oviposition; S-diet - II oviposition; (S+P)-diet – I oviposition; (S+P)-diet – II oviposition. Boxplots and bar-charts of each distribution were generated.

To investigate if the variability of the analyzed parameters was linked with some variables such as sex, diet type, treatment concentration, time of the oviposition test, analysis of covariance (ANCOVA) was performed using the above-mentioned variables as covariates. A General Linear Model (GLM) was generated for each compound with a p-value for each variable and the

model summary consisting of R^2. R^2 adjusted and S, to analyze how well the model fitted the data. Even when the summary of the model was not robust, if the p-value of a variable was ≤ 0.05 it was taken as an indication that that variable was linked with the variability of the analyzed parameter.

In the datasets where diet type was underlined as a covariate that significantly influenced the parameters, a further ANCOVA was carried out on the dataset of each diet type in order to verify whether the others variables were influent within the same diet experiment.

For each of the analyzed parameter, One-way analysis of variance was carried out verify if there were statistical differences between any of the mean of the dataset. Levene's test for homogeneity of variance from means and medians was performed to assess if the assumption of homoscedasticity, required by ANOVA, was verified and the H0 was accepted with p-value ≥ 0.05%.

In some cases, datasets were transformed using Box-Cox transformation or Log transformation to achieve homoscedasticity and normality distribution of the residuals. Subsequently, One-way ANOVA was performed. The normality distribution of the residual was verified with the Shapiro-Wilk test with a W ≥ 0.05 and a p normal ≥ 0.05. Tukey's pairwise post-hoc test was used to identify the treatments that were statistically different and Dunnet simultaneous test for level mean versus control mean revealed the treatments that statistically differ from the control.

3.3.2 MOLECULAR DATA

The logarithm of the fold change $2^{-(\Delta\Delta Ct)}$ was used as an index of the relative abundance of the bacterial titer in comparison with the control. The dataset was divided into 4 subsets by organ (oesophageal bulb and mid-gut) and diet type. The distributions of datasets were analyzed and the summary statistic calculated. Successively, the normal distribution of these subsets was verified with a Shapiro-Wilk test with a W ≥ 0.05 and a p normal ≥ 0.05. Bar charts were generated for each subset.

One-way ANOVA was performed on each dataset separately to verify if the bacterial titer detected from the real-time PCR at each treatment significantly differ from the control. Levene's test for homogeneity of variance from means and medians was performed to assess if the

assumption of homoscedasticity, required by ANOVA, was verified and the H_0 was accepted with p-value $\geq$ 0.05%.

Tukey's pairwise post-hoc test was used to identify the treatments that were statistically different and Dunnet simultaneous test for level mean versus control mean revealed the treatments that statistically differ from the control.

ANCOVA was performed to verify if the variables diet type, treatment concentration, and organ type were significantly influent on the bacterial titer variation.

A GLM was generated for each compound with a p-value for each variable and the model summary consisting of R^2. R^2 adjusted and S, to analyze how well the model fits the data.

4 RESULTS

4.1 TEST OF FITNESS REDUCTION THROUGH MICROBIOME ALTERATION

The results of fitness tests showed statistically significant differences among a large part of the treatments and the control for most of the analyzed parameters.

4.1.1 MORTALITY

The mortality rates at the 14th day of trial for each treatment are presented in Table 4. The dataset distribution is shown in two Boxplots, separately for S-diet and (S+P)-diet (Figure 4 and Figure 6).

Table 4: Mean mortality rates recorded at the 14th day of treatment ± SD.

Treatment	Diet	n° specimens/ group	n° repetition	total n° specimens	mean rate of mortality on the 14th day of treatment ± SD	
					♀	♂
CONTROL	S	50	5	250	28% ± 16.7	27% ± 12.1
CONTROL	S+P	50	4	200	26% ± 17.4	36.5% ± 25.7
ANTIBIOTICS 0.01‰	S	50	3	150	32.8% ± 12.7	31% ± 23.9
ANTIBIOTICS 0.01‰	S+P	50	3	150	28.3% ± 4.5	53.3 ± 19
COPPER OX. 0.5%	S	50	3	150	46% ± 10.5	51.5 ± 10.6
COPPER OX. 0.5%	S+P	50	3	150	95% ± 5.1	91.6 ± 4.1
COPPER OX. 0.1%	S	50	3	150	41% ± 2	31.3% ± 10
COPPER OX. 0.1%	S+P	50	3	150	75% ± 9.6	74% ± 5.5
COPPER OX. 0.02%	S	50	3	150	21.3 % ± 10.7	15 ± 8.8
COPPER OX. 0.02%	S+P	50	3	150	24.6% ± 22.8	26.6% ± 11
VIRIDIOL 0.5%	S	50	3	150	18% ± 11.5	24.6% ± 13.5
VIRIDIOL 0.5%	S+P	50	3	150	26% ± 4.5	94± 5.5
VIRIDIOL 0.1%	S	50	3	150	10% ± 2.6	24% ± 11.1
VIRIDIOL 0.1%	S+P	50	3	150	12.6% ± 7.3	95.6 ± 5.8
VIRIDIOL 0.05%	S+P	50	3	150	53% ± 17.7	69.6% ± 14.7
HARZIANIC ACID 0.5%	S	50	3	150	14.3 ± 9	14.6 ± 9
HARZIANIC ACID 0.5%	S+P	50	3	150	34.6 ± 8.5	72 ± 24.9
HARZIANIC ACID 0.1%	S	50	3	150	8.6 ± 3.5	12% ± 3.6
HARZIANIC ACID 0.1%	S+P	50	3	150	34% ± 9.8	28.3 ± 5
HARZIANIC ACID 0.05%	S+P	50	3	150	25% ± 6	22.3% ± 6.5
6-PENTYL-α-PYRON 5%	S	50	3	150	37% ± 21.3	46.6% ± 21
6-PENTYL-α-PYRON 5%	S+P	50	3	150	74.6% ± 8.3	56.3% ± 6.1
6-PENTYL-α-PYRON 1%	S	50	3	150	13.6% ± 9.3	23% ± 11.7

6-PENTYL-α-PYRON 1%	S+P	50	3	150	32.3% ± 12.9	31% ± 11
LIPOPEPTIDES 5%	S	50	3	150	15.6 ± 13.6	52 ± 12.7
LIPOPEPTIDES 5%	S+P	50	3	150	92% ± 7.5	94% ± 4.7
LIPOPEPTIDES 1%	S	50	3	150	23% ± 9.5	47 ± 11.5
LIPOPEPTIDES 1%	S+P	50	3	150	43% ± 8.3	92.3% ± 7

The statistical analysis results showed that diet type was significantly influent on all the administered compounds, with exception of Control and Antibiotics treatment. In some cases, where diet was a covariate, also sex was significantly influent. The concentration was also detected as a covariate for some compounds.

With regard to the **Control** and **Antibiotics** treatment, the results showed very low values of R^2 and R^2 adjusted with a high value of S, which assume that the model does not fit well the data. P values were all ≥ 0.05 either for sex and diet variable which means that the mortality variability in these treatments is not explained and linked with the examined variables. Concentration was not included as a variable since there was no difference in concentration into the experiment program both for antibiotics and control.

The GLM generated for **Copper Oxychloride** evidenced a value of $R^2 = 90.6$ %, an adjusted $R^2 = 89.1\%$ with S = 7.8. Both diet type and concentration were considered as covariates (p-value $_{\text{diet type}} = 0.000$; p-value $_{\text{concentration}} = 0.000$) while sex variable was discarded (p-value $_{\text{sex}} = 0.626$). The variance inflation factors of both the covariates were very low (VIF $_{\text{diet type}} = 1.00$; VIF $_{\text{concentration}} = 1.33$), diet type and concentration resulted as independent variables and that the diet type is the most influent variable in flies' mortality. Thus, the rate of mortality in flies treated with Copper Oxychloride is significantly higher in (S+P)-diet and is directly proportional to the concentration of the treatment administered, with no significant difference between sexes.

The GLM generated for **Viridiol** evidenced a value of $R^2 = 69.5\%$, an adjusted $R^2 = 64.6\%$ with S = 19. Both diet type and sex were considered as covariates (p-value $_{\text{diet type}} = 0.000$. p-value $_{\text{sex}} = 0.000$) while concentration variable was discarded (p-value $_{\text{concentration}} = 0.804$). The variance inflation factors of both the covariates were very low (VIF $_{\text{diet type}} = 1.30$; VIF $_{\text{sex}} = 1.00$), therefore sex of the flies and concentration are independent variables and that sex is the most influent variable in flies' mortality. The mortality rate in flies treated with Viridiol is significantly higher in male and the combination of the treatment with the (S+P)-diet increases the mortality with no significant influence of the concentration.

The GLM generated for **Harzianic Acid** evidenced a value of R^2 = 60.1%, an adjusted R^2 = 53.74% and an S = 13. Both diet type and concentration were considered as covariates (p-value $_{\text{diet type}}$ = 0.000; p-value $_{\text{concentration}}$ = 0.005) while sex variable was discarded (p-value $_{\text{sex}}$ = 0.198). The variance inflation factors of both the covariates were very low (VIF $_{\text{diet type}}$ = 1.20; VIF $_{\text{concentration}}$ = 1.75), therefore diet type and concentration are independent variables and that the diet type is the most influent variable in flies' mortality. The mortality rate in flies treated with Harzianic Acid is significantly higher in (S+P)-diet and is directly proportional to the concentration of the treatment administered, with no significant difference between sexes.

The GLM generated for **6-pentyl-α-pyron** evidenced a value of R^2 = 63.7%, an adjusted R^2 = 58.2% with S = 14. Both diet type and concentration were considered as covariates (p-value $_{\text{diet type}}$ = 0.004; p-value $_{\text{concentration}}$ = 0.000) while sex variable was discarded (p-value $_{\text{sex}}$ = 0.989). The variance inflation factors of both the covariates were very low (VIF $_{\text{diet type}}$ = 1.00; VIF $_{\text{concentration}}$ = 1.00) the diet type and concentration are independent variables and that are equally influent variables on flies' mortality. The mortality rate in flies treated with Harzianic Acid is significantly higher in (S+P)-diet and is directly proportional to the concentration of the treatment administered, with no significant difference between sexes.

The GLM generated for **Lipopeptides** extracted from *B. subtilis* evidenced a value of R^2 = 80.9 %, an adjusted R^2 = 78.09 % with S = 14. Both diet type and sex were considered as covariates (p-value $_{\text{diet type}}$ = 0.000. p-value $_{\text{sex}}$ = 0.000) while concentration variable was discarded (p-value $_{\text{concentration}}$ = 0.058). The variance inflation factors of both the covariates were very low (VIF $_{\text{diet type}}$ = 1.00; VIF $_{\text{sex}}$ = 1.00), therefore the sex and the concentration are independent variables and equally influent variables on mortality. The mortality rate in flies treated with Lipopeptides is significantly higher in male flies and the combination of the treatment with the (S+P)-diet increases the mortality with no significant influence of the concentration.

In S-diet dataset, Levene's test confirmed the homogeneity of variance from means and medians (p-value means = 0.552; p-value medians = 0.894). The normality of the distribution was verified with the Shapiro-Wilk test (W=0.976; p normal = 0.591). One-Way ANOVA performed on diet S dataset evidenced that some of the means significantly differ from other in both sexes.

Tukey's pairwise post-hoc test and Dunnet test showed no significant differences between the control and the treatments mortality in both sexes at all the treatment concentration. A significant difference was evinced between Copper Oxychloride 0.5% and Copper Oxychloride 0.02%

treatments (p-value = 0.015), underlining that the concentration of this compound deeply affects the flies' mortality and that the higher concentration led to a significantly higher mortality (Figure 5).

ANOVA detected a significant difference between females and males' mortality in Lipopeptides 5% treatment (p-value = 0.028), showing a higher mortality in males than in females.

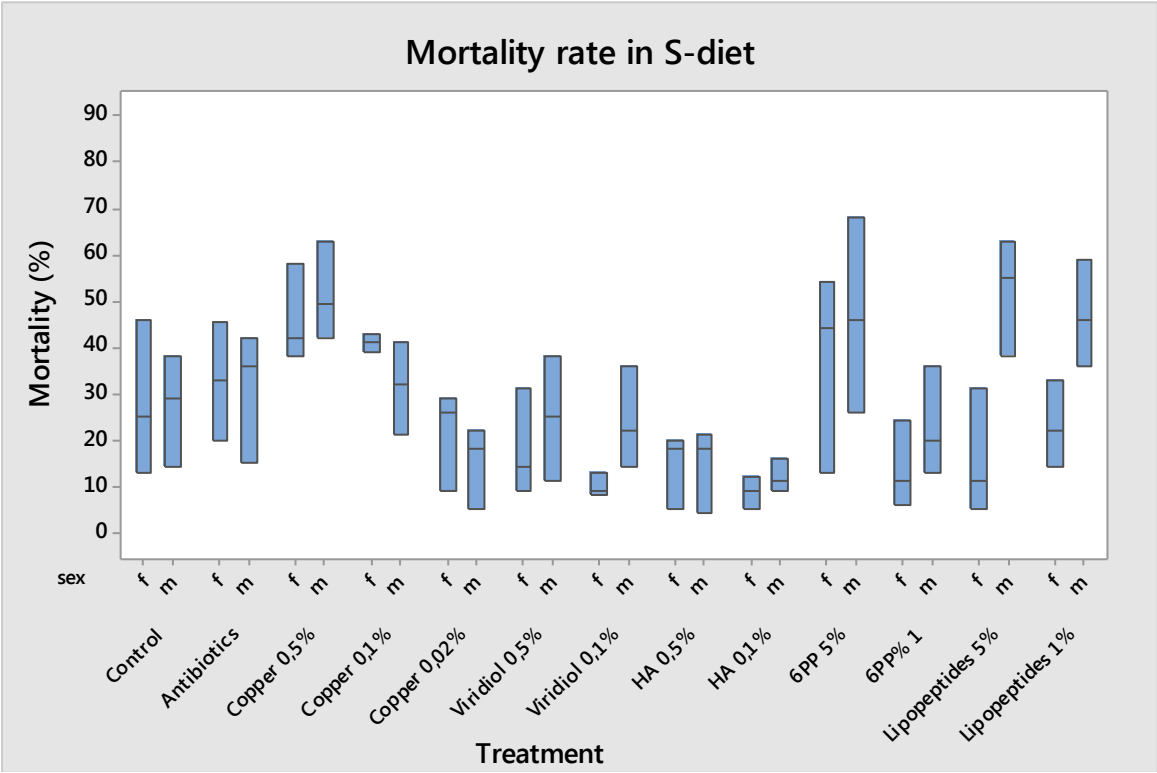

Figure 4: Boxplot of mortality rate recorded at each treatment in females and males fed on S-diet

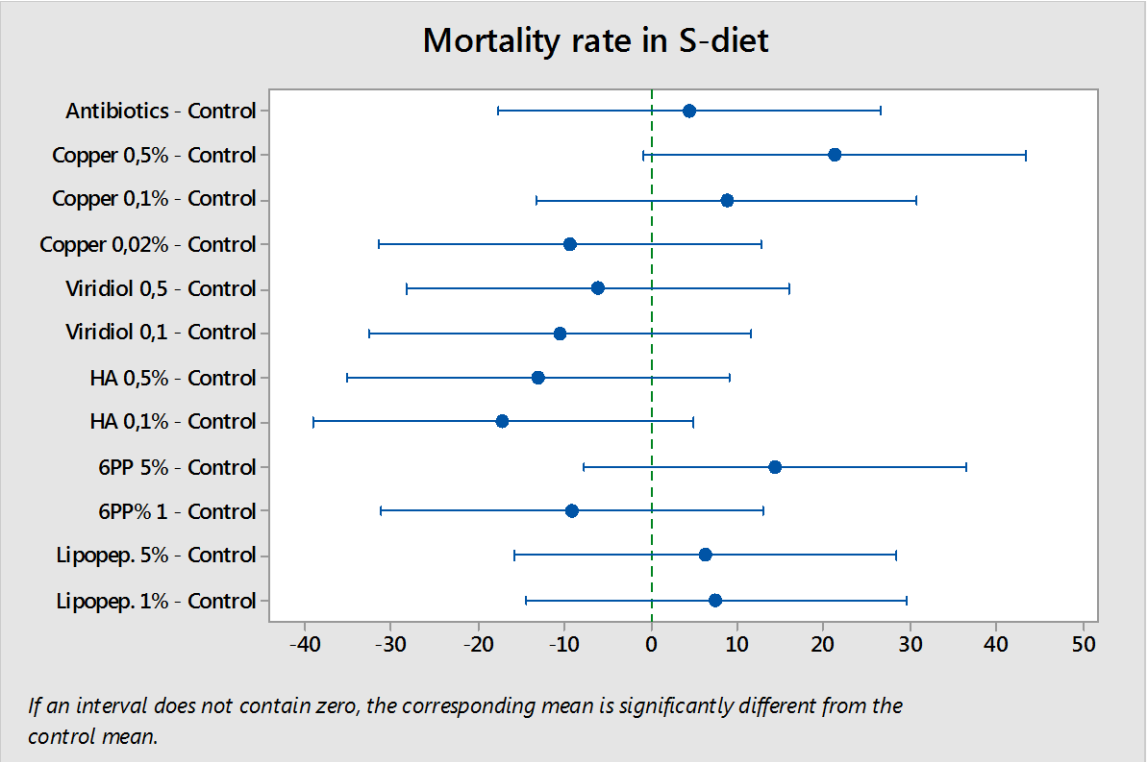

Figure 5: *Dunnet test result on mortality rate recorded at each treatment in flies fed on S-diet*

With regard to the (S+P)-diet, ANCOVA on (S+P)-diet dataset showed that sex was significantly influent on mortality in some treatments, therefore, two ANOVA were performed on the female's and male's datasets separately and the results showed that some of the means significantly differ from other in both sexes.

Tukey's pairwise post-hoc test identified the treatments that were statistically different and Dunnet simultaneous test revealed the treatments that statistically differed from the control (Figure 7 and Figure 8).

The mortality rate recorded in **Copper Oxychloride** treatments at a concentration of 5% and 1% and in Lipopeptides treatment at a concentration of 5% was significantly higher in both sexes in comparison with the control (p value = 0.000). The mortality rate recorded in 6-pentyl-α-pyron 5% treatment was significantly higher only for females (p value = 0.001). The mortality rate recorded in Viridiol treatments at all the concentrations ($p\ value_{VIR\ 0.5\%} = 0.000$; $p\ value_{VIR\ 0.1\%} = 0.000$; $p\ value_{VIR\ 0.05\%} = 0.011$), in Harzianic Acid 5% ($p\ value_{HA\ 0.05\%} = 0.018$) and Lipopeptides treatment at a concentration of 1% ($p\ value_{LIP\ 5\%} = 0.00$) was significantly higher only for males.

Significant differences between different concentrations of the same administered compound were also evidenced in both sexes. In females' subset, the mortality was significantly higher in 6-pentyl-α-pyron 5% treatment in comparison with the less concentrated 6-pentyl-α-pyron 1% (p-value = 0.005) and the same trend was recorded for the Lipopeptides treatment (p-value = 0.000). On the contrary, a significantly higher mortality was recorded in the less concentrated Viridiol treatment (p-value $_{\text{VIR 0.05\%}}$ = 0.045) in comparison with the 0.1% concentration but not with the most concentrated one, the 0.5%. In males' subset, the mortality rate was significantly higher in the most concentrated treatment of Harzianic Acid (HA 0.5%) in comparison with both the less concentrated treatments (HA 0.1% with p-value = 0.014; HA 0.05% with p-value = 0.003). In both the subsets the most concentrated treatment of Copper Oxychloride led to a significantly higher mortality in comparison with both the less concentrated treatments (CO 0.1% with p-value = 0.011; CO 0.02% with p-value = 0.000). These analyses confirmed that the concentration has a significant influence on the flies' mortality when combined with (S+P)-diet.

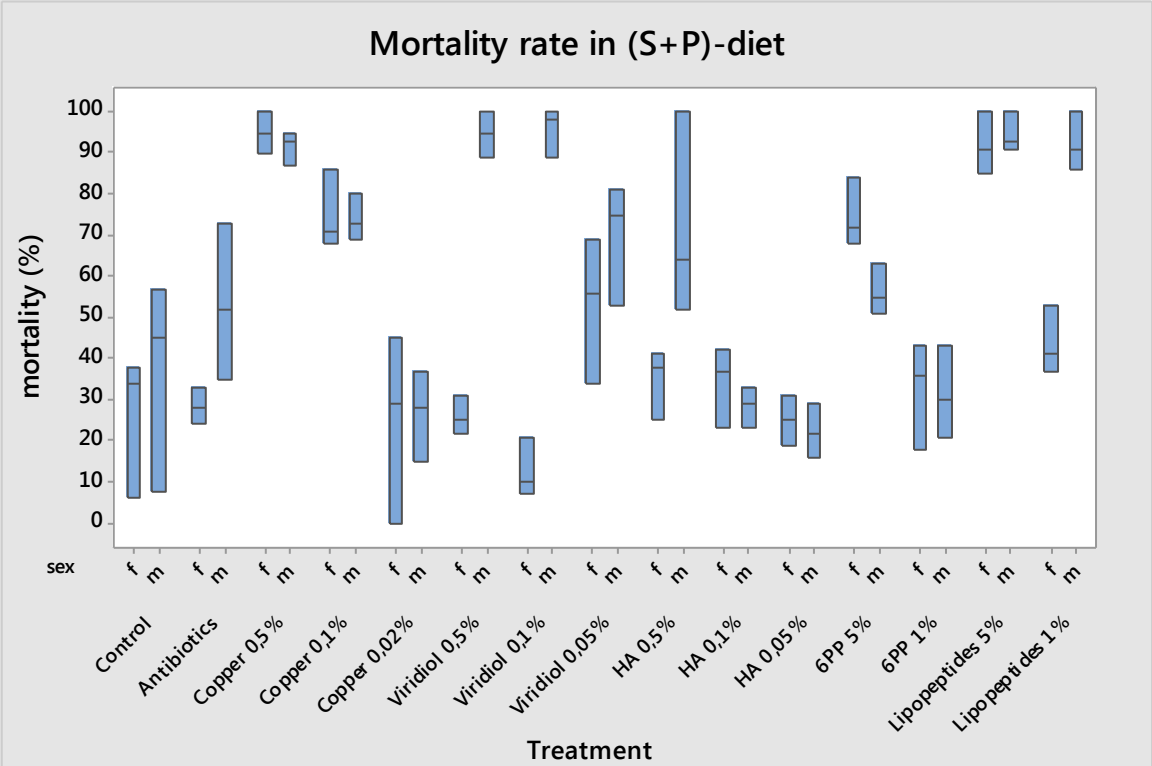

Figure 6: *Boxplot of the mortality rate recorded at each treatment in females and males fed on (S+P)-diet*

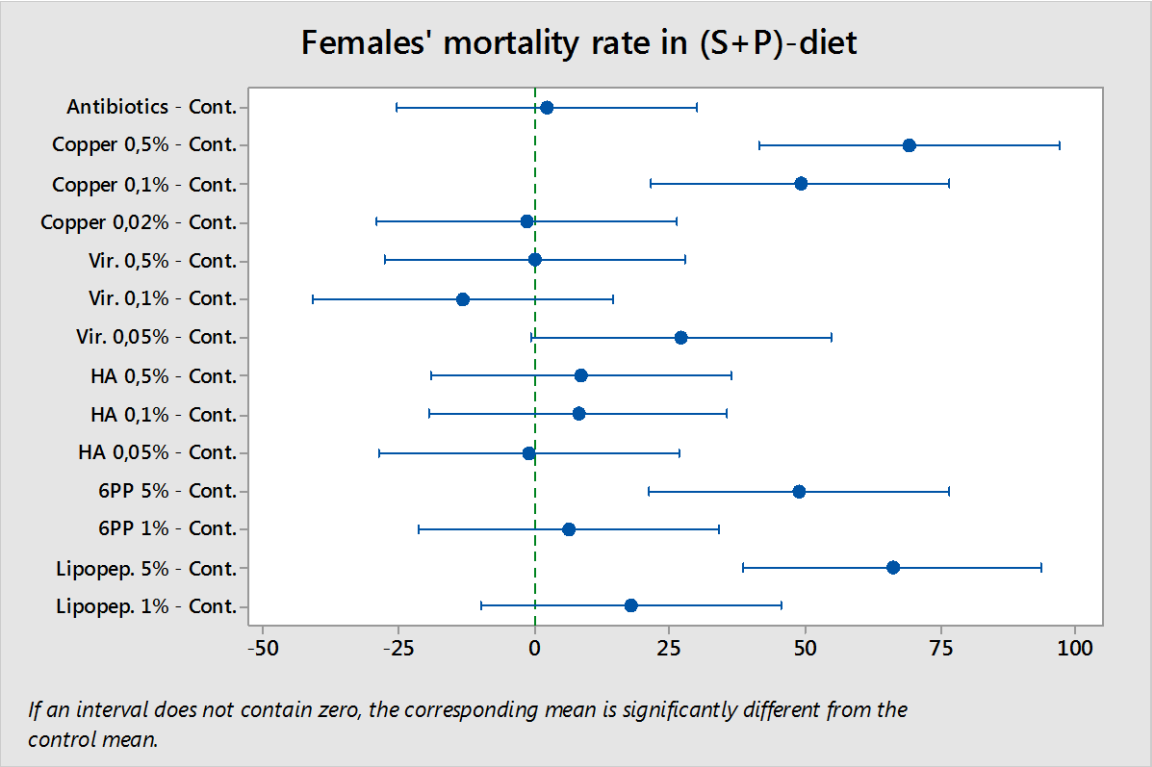

Figure 7: *Dunnet test result on the mortality rate recorded at each treatment in females fed on S -diet*

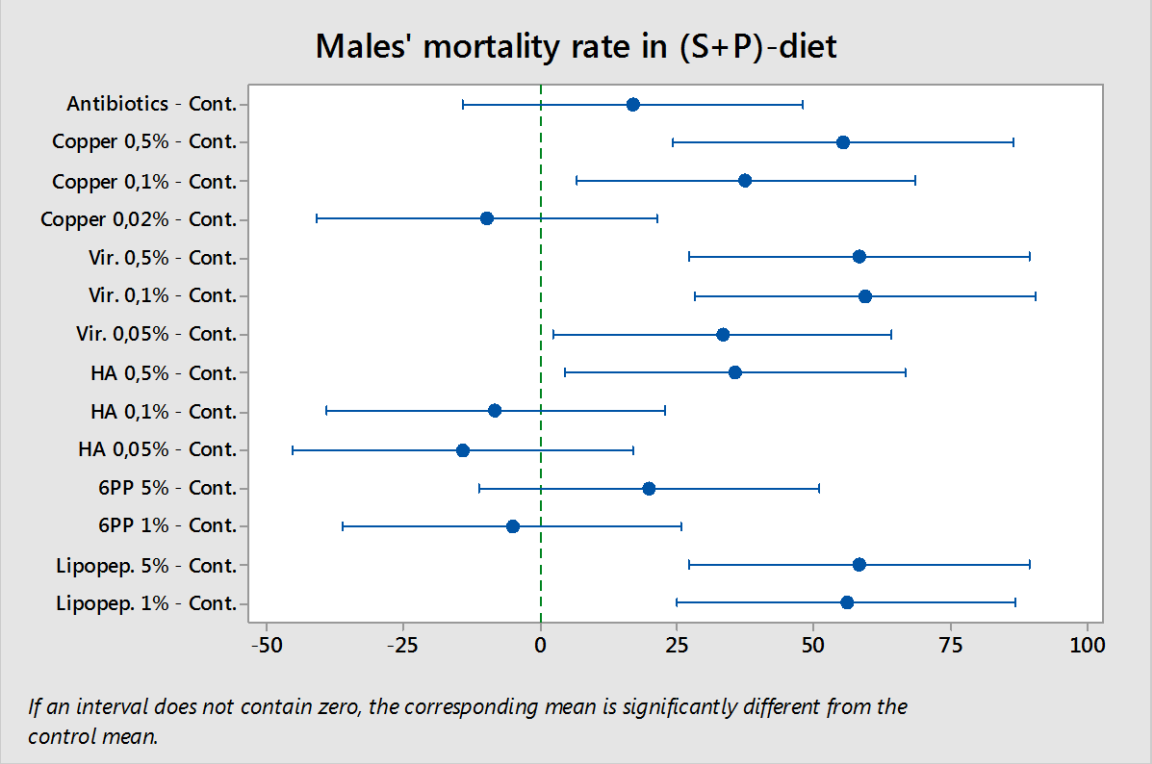

Figure 8: *Dunnet test result on the mortality rate recorded at each treatment in males fed on S-diet*

4.1.2 DAILY DIET CONSUMPTION

Daily ingested diet per specimen was used also to verify if some of the treatments were repellent for flies. The recorded data and the summary statistic are reported in Table 5. The dataset distribution is shown in two Boxplots, separately for S-diet and (S+P)-diet (Figure 9 and Figure 10).

Table 5: Mean of the daily diet consumption of females and males at each treatment calculated in mg per specimen per day ± Standard deviation

Treatment	Diet	n° specimens/ group	n° repetition	Total n° specimens	Mean quantity of diet ingested ± SD (mg/day/specimen)	
					♀	♂
CONTROL	S	50	5	250	0.641±0.052	0.536 ± 0.083
CONTROL	S+P	50	4	200	0.606±0.140	0.516 ± 0.134
ANTIBIOTICS 0.01‰	S	50	3	150	0.631 ± 0.011	0.603 ± 0.028
ANTIBIOTICS 0.01‰	S+P	50	3	150	0.607±0.032	0.612 ± 0.032
COPPER OX. 0.5%	S	50	3	150	0.449 ± 0.028	0.316 ± 0.021
COPPER OX. 0.5%	S+P	50	3	150	0.709±0.056	0.480 ± 0.073
COPPER OX. 0.1%	S	50	3	150	0.530 ± 0.038	0.454 ± 0.050
COPPER OX. 0.1%	S+P	50	3	150	0.330±0.012	0.175 ± 0.005
COPPER OX. 0.02%	S	50	3	150	0.646 ± 0.020	0.541 ± 0.024
COPPER OX. 0.02%	S+P	50	3	150	0.627±0.047	0.585 ± 0.027
VIRIDIOL 0.5%	S	50	3	150	0.535 ± 0.036	0.458 ± 0.031
VIRIDIOL 0.5%	S+P	50	3	150	0.600±0.020	0.506 ± 0.022
VIRIDIOL 0.1%	S	50	3	150	583 ± 0.048	0.536 ± 0.041
VIRIDIOL 0.1%	S+P	50	3	150	0.603±0.017	0.614 ± 0.056
VIRIDIOL 0.05%	S+P	50	3	150	0.642±0.051	0.591 ± 0.031
HARZIANIC ACID 0.5%	S	50	3	150	0.607 ± 0.034	0.506 ± 0.081
HARZIANIC ACID 0.5%	S+P	50	3	150	0.638±0.088	0.593 ± 0.159
HARZIANIC ACID 0.1%	S	50	3	150	0.665 ± 0.062	0.604 ± 0.016
HARZIANIC ACID 0.1%	S+P	50	3	150	0.727±0.184	0.635 ± 0.102
HARZIANIC ACID 0.05%	S+P	50	3	150	0.711±0.029	0.703 ± 0.028
6-PENTYL-A-PYRON 5%	S	50	3	150	0.474 ± 0.055	0.415 ± 0.069
6-PENTYL-A-PYRON 5%	S+P	50	3	150	0.351±0.031	0.401 ± 0.012
6-PENTYL-A-PYRON 1%	S	50	3	150	0.648 ± 0.049	0.540 ± 0.060
6-PENTYL-A-PYRON 1%	S+P	50	3	150	0.607±0.043	0.615 ± 0.064
LIPOPEPTIDES 5%	S	50	3	150	0.494 ± 0.040	0.425 ± 0.035
LIPOPEPTIDES 5%	S+P	50	3	150	0.551±0.058	0.491 ± 0.039
LIPOPEPTIDES 1%	S	50	3	150	0.646 ± 0.034	0.560 ± 0.044
LIPOPEPTIDES 1%	S+P	50	3	150	0.607±0.035	0.555 ± 0.029

Statistical analysis results showed that the treatment concentration was significantly influent on the quantity of diet ingested by flies in most of the treatments with an inversely proportional function, while the diet type seems to be irrelevant in most of the treatment. Sex resulted as deeply influent on most of the treatment including Control and Antibiotics, thus it seems that female flies have naturally the tendency to eat more, independently from the treatment and the diet type, probably because of the nutritional needs required for oogenesis.

With regard to the Control and Antibiotics treatment, the results showed very low values of R^2 and adjusted R^2 with a high value of S, which assume that the model does not fit well the data. The p-value for diet type was ≥ 0.05 which means that this variable is not linked with the variability of the ingested diet in control and antibiotics dataset. On the contrary, sex was evidenced as significantly influent (p-value $_{ANTIB}$ = 0.047; p-value $_{CONT}$ = 0.004), with a significantly higher quantity of diet ingested by females in comparison with males. Concentration was not included as a variable since there was no difference in concentration into the experiment program for both antibiotics and control.

The GLM generated for **Copper Oxychloride** evidenced a value of R^2 = 64.51 %, an adjusted R^2 = 58.63% with S = 43. Both sex and concentration were considered as covariates (p-value $_{sex}$ = 0.000; p-value $_{concentration}$ = 0.000) while diet type variable was discarded (p-value $_{diet\ type}$ = 0.896). The variance inflation factors of both the covariates were very low (VIF $_{sex}$ = 1.00; VIF $_{concentration}$ = 1.33), sex and concentration are independent variables and the first parameter is the most influent variable on the quantity of diet ingested by flies when copper is added. Thus, the quantity of diet ingested by flies treated with Copper Oxychloride is significantly lower in males and the more the treatment is concentrated the less the flies tend to ingest it.

The GLM generated for **Viridiol** evidenced a value of R^2 = 66.5%, an adjusted R^2 = 61.2% with S = 39. All the three variables: concentration, diet type and sex, were considered as covariates (p-value $_{diet\ type}$ = 0.003; p-value $_{sex}$ = 0.001; p-value $_{concentration}$ = 0.002). The variance inflation factors of all the covariates were very low (VIF $_{diet\ type}$ = 1.20; VIF $_{sex}$ = 1.00; VIF $_{concentration}$ = 1.87), thus the three variables resulted independent and sex is the most influent variable on daily diet consumption. Therefore, the amount of ingested diet by flies treated with Viridiol is significantly lower in males and in S-diet experiments and it is inversely proportional to treatment concentration.

The GLM generated for **Harzianic Acid** evidenced a value of R^2 = 36.8%, an adjusted R^2 = 26.6% and an S = 87. The variables, concentration, diet type and sex were discarded as covariates with p values ≥ 0.05 (p-value $_{\text{diet type}}$ = 0.065; p-value $_{\text{sex}}$ = 0.153; p-value $_{\text{concentration}}$ = 0.071). Therefore, the amount of ingested diet is not affected by sex, diet type, and treatment concentration.

The GLM generated for **6-pentyl-α-pyron** evidenced a value of R^2 = 75.3%, an adjusted R^2 = 71.7% with S = 61.1. Concentration was verified as covariate (p-value $_{\text{concentration}}$ = 0.000) while sex and diet type variables were discarded (p-value $_{\text{diet type}}$ = 0.317; p-value $_{\text{sex}}$ = 0.293). The variance inflation factor of concentration was very low (VIF $_{\text{concentration}}$ = 1.00). Therefore, the daily diet intake in flies treated with 6-pentyl-α-pyron is related with the treatment concentration and resulted inversely proportional to this parameter.

The GLM generated for **Lipopeptides** extracted from *B. subtilis* evidenced a value of R^2 = 70.7 %, an adjusted R^2 = 66.3 % with S = 43. Both treatment concentration and sex were considered as covariates (p-value $_{\text{concentration}}$ = 0.000. p-value $_{\text{sex}}$ = 0.002) while diet type variable was discarded (p-value $_{\text{concentration}}$ = 0.896). The variance inflation factors of both the covariates were very low (VIF $_{\text{diet type}}$ = 1.00; VIF $_{\text{sex}}$ = 1.00), with sex and treatment concentration independent variables equally influent on the amount of ingested diet. Thus, the daily diet intake in flies treated with Lipopeptides is significantly lower in males and it is inversely proportional to treatment concentration.

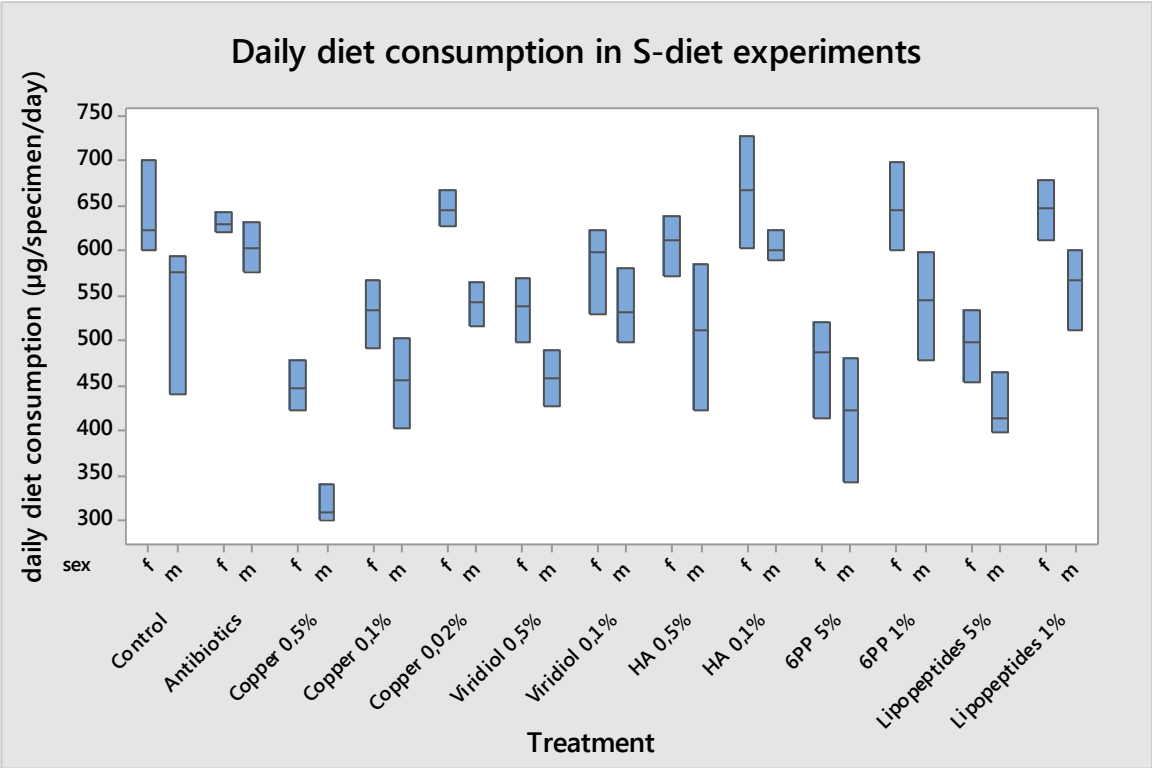

Figure 9: *Boxplot of daily diet consumption of females and males fed on S-diet calculated in µg per specimen per day*

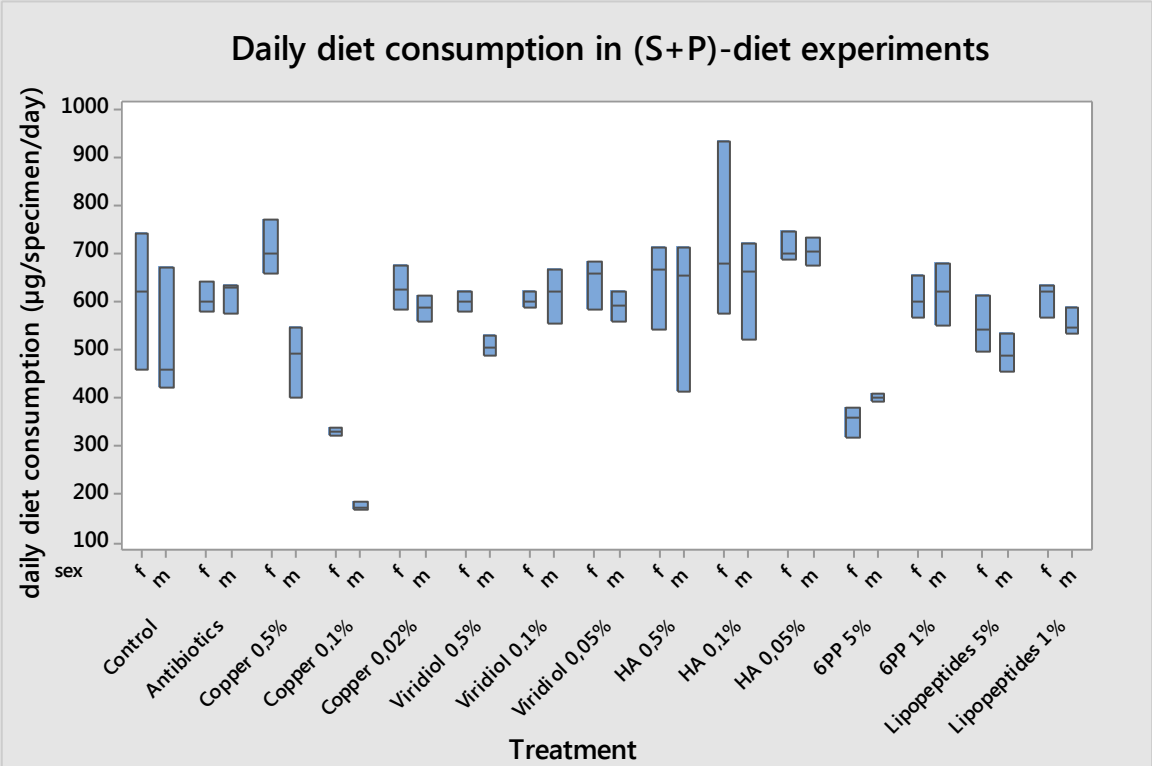

Figure 10: *Boxplot of daily diet consumption of females and males fed on (S+P)-diet calculated in µg per specimen per day*

Observing boxplots obtained from the distribution of the two different diet datasets (Fig. 8-9), it seems that two different patterns are displayed. Analyzing these datasets separately by ANCOVA with sex and treatment concentration as variables this hypothesis is confirmed. With regards to S-diet dataset, sex and concentration were evidenced as significant variables on the quantity of ingested diet with p values ≤ 0.05 in all the treatments including Control and Antibiotics. The analysis clearly showed that males fed significantly less than females and that there is an inversely proportional correlation between daily intake and the treatment concentration: the more the treatment is concentrated the less the flies tend to feed. On the other hand, (S+P)-diet dataset showed a less clear pattern with Control and Antibiotic treatment data that evidenced no significant difference between sex, Harzianic Acid treatment data that seems to be independent from both sex and concentration and 6-pentyl-α-pyron treatment data whose variability showed no correlation with treatment concentration.

With regards to the S-diet, the influence of sex on the variability was recorded in all treatments. Levene's test confirmed the homogeneity of variance from means and medians (p-value means = 0.691; p-value medians = 0.820). The normality of the distribution was verified with the Shapiro-Wilk test (W=0.983; p normal = 0.564). The ANOVA carried out on both sexes showed that the daily intake in flies treated with Copper Oxychloride 0.5%, 6-pentyl-α-pyron 5% and Lipopeptides 5% were statistically different from the Control one with p values ≤ 0.05 (Figure 11). Significant differences between different concentrations of the same administered compound were also recorded. The quantity of ingested diet was significantly lower in 6-pentyl-α-pyron 5% treatment in comparison with the less concentrated 6-pentyl-α-pyron 1% (p-value = 0.006) and the same trend was recorded for the Lipopeptides dataset between 5% and 1% treatments (p-value = 0.010) and Copper Oxychloride dataset between 0.5% and 0.02% treatment (p-value =0.000).

With regards to the (S+P)-diet, the sex influence on the variability was not recorded in all treatments. The ANOVA carried out on females' and males' dataset separately showed that the daily diet intake in flies treated with Copper Oxychloride 0.1% was significantly lower in both females and males while in 6-pentyl-α-pyron 5% it was statistically lower only in females' subset with p values ≤ 0.05 (Figure 12 and Figure 13). Significant differences between different concentrations of the same administered compound were also evidenced. In females' subset, Copper Oxychloride 0.1% treatment was showed as statistically different from highest

concentration: 0.5% (p-value = 0.000); and the lowest concentration: 0.02 (p-value =0.029), underlying that the females treated with Copper Oxychloride 0.1% fed significantly less in comparison with the other two treatment concentration. In males' subset, 6-pentyl-α-pyron 5% was evidenced as statistically different from the lower concentration 1%, thus males treated with the most concentrated treatment tend to feed less than the counterparts treated with the last concentrated diet.

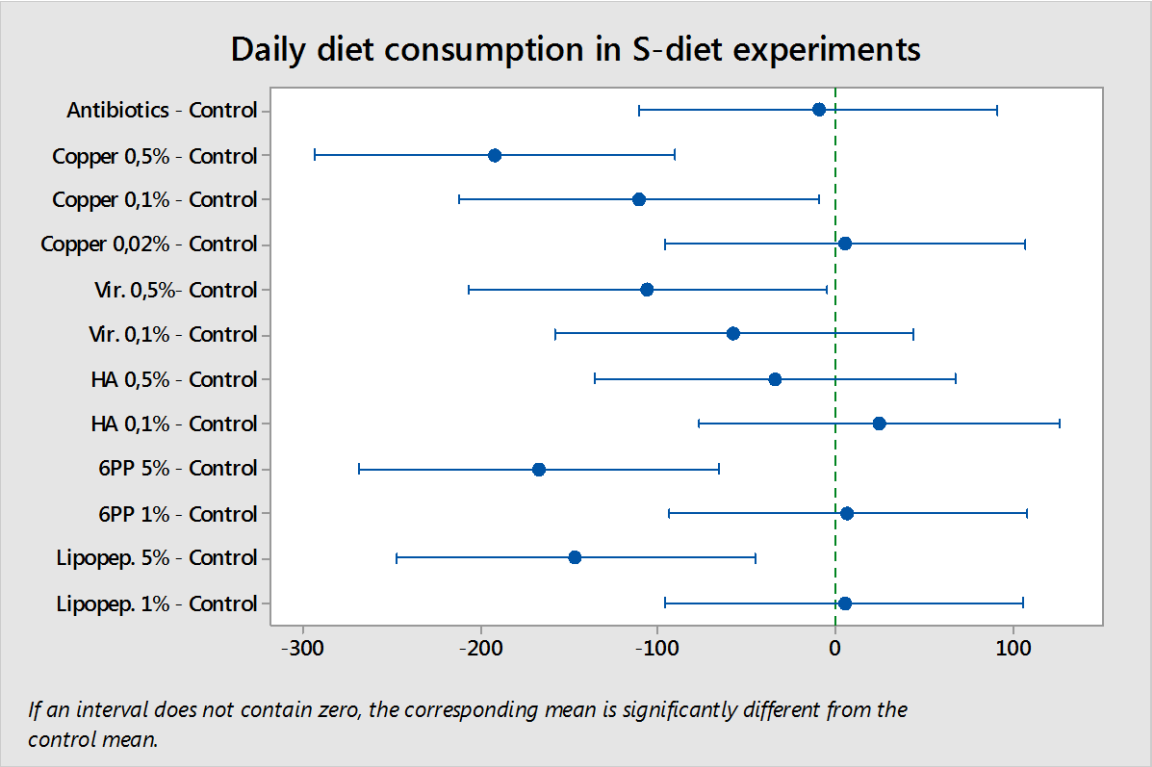

Figure 11: Dunnett test result of daily diet consumption of flies fed on S-diet

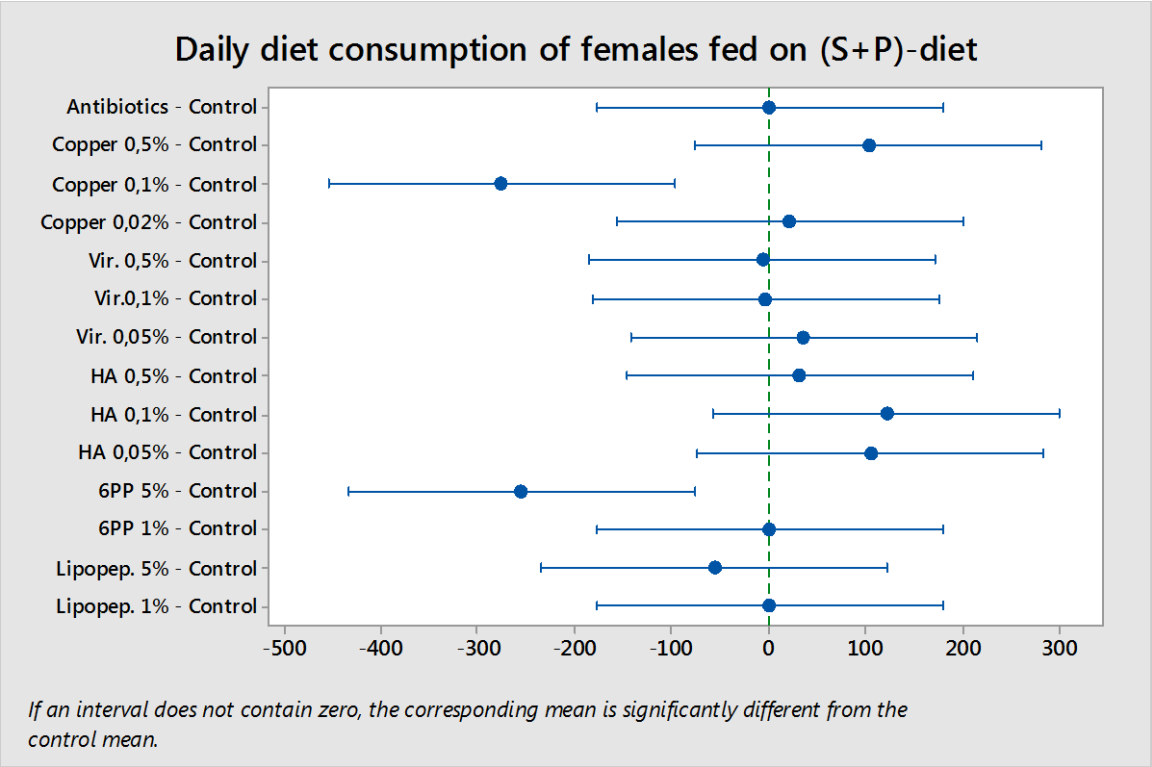

Figure 12: Dunnett test result of daily diet consumption of females fed on (S+P)-diet

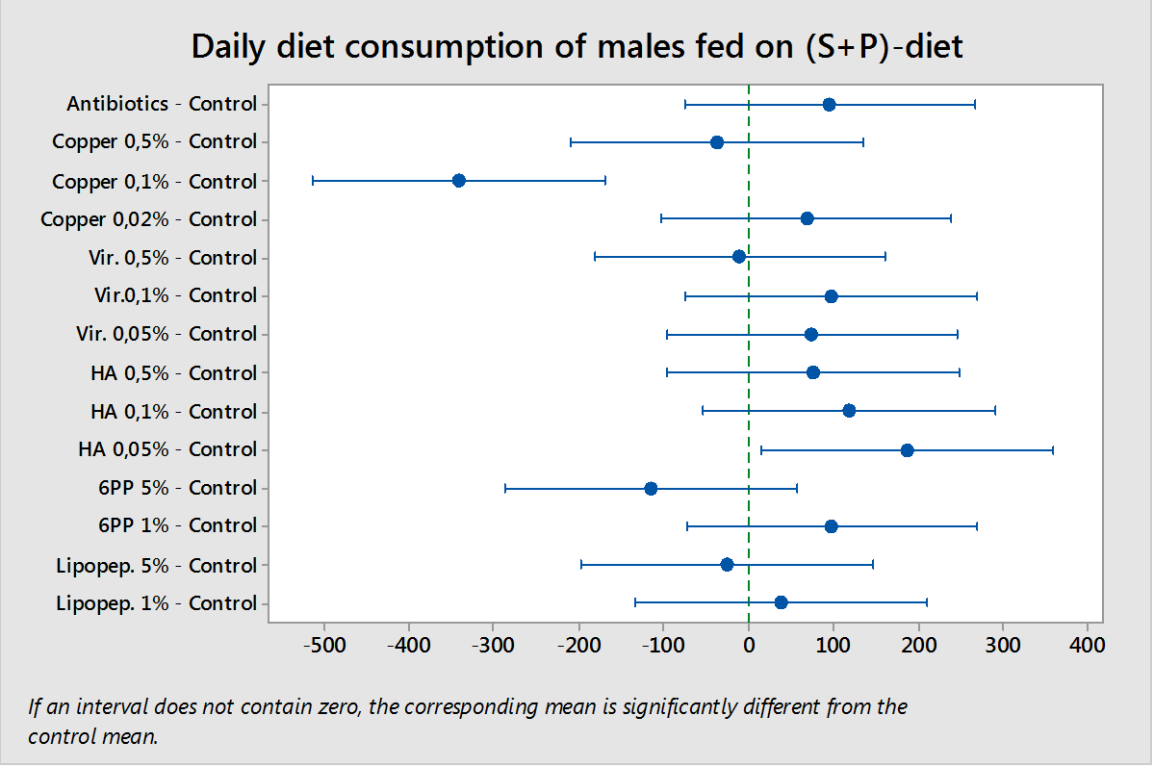

Figure 13: Dunnett test result of daily diet consumption of males fed on (S+P)-diet

4.1.3 FECUNDITY

The average number of eggs laid per female was used as an adult fitness indicator. The data of some treatments on (S+P)-diet were not shown because of the high rate of mortality in one or both sexes (higher than 90%) that made impossible to conduct all the fitness test due to the lack of available specimens. The recorded data and the summary statistic are presented in Table 6. The dataset distribution is shown in two Boxplots, separately for S-diet and (S+P)-diet (Figure 14 and Figure 15).

Table 6: Mean number of eggs laid by flies at each treatment ± Standard deviation in the first and second oviposition test and in total

Treatment	Diet	n° specimens/ group	n° repetition	Total n° specimens	Mean number of laid eggs/female ± SD		
					I oviposition	II oviposition	Total
CONTROL	S	50	5	250	1.23±1.26	2.38 ± 1.51	3.65 ± 2.22
CONTROL	S+P	50	4	200	5.87±3.12	3.633 ± 0.90	9.50 ± 4.02
ANTIBIOTICS 0.01‰	S	50	3	150	1.15±0.60	1.71 ± 0.34	2.86 ± 0.92
ANTIBIOTICS 0.01‰	S+P	50	3	150	5.11±1.66	3.46 ± 0.85	8.47 ± 2.51
COPPER OX. 0.5%	S	50	3	150	0.01 ± 0.01	0.03 ± 0.01	0.04 ± 0.02
COPPER OX. 0.5%	S+P	50	3	150			
COPPER OX. 0.1%	S	50	3	150	0.07 ± 0.02	0.12± 0.09	0.17± 0.08
COPPER OX. 0.1%	S+P	50	3	150	0.04±0.03	0.004 ± 0.004	0.044 ± 0.05
COPPER OX. 0.02%	S	50	3	150	1.43± 1.31	0.62 ± 0.45	2.05 ± 1.76
COPPER OX. 0.02%	S+P	50	3	150	0.06±0.04	0.013± 0.011	0.073± 0.046
VIRIDIOL 0.5%	S	50	3	150	1.21 ± 0.30	1.33 ± 0.90	2.54 ± 1.06
VIRIDIOL 0.5%	S+P	50	3	150			
VIRIDIOL 0.1%	S	50	3	150	0.26 ± 0.20	1.66± 0.71	1.93± 0.72
VIRIDIOL 0.1%	S+P	50	3	150			
VIRIDIOL 0.05%	S+P	50	3	150	1.06±0.25	0.89 ± 0.21	1.96 ± 0.20
HARZIANIC ACID 0.5%	S	50	3	150	1.95 ± 0.22	1.04 ± 0.27	2.99 ± 0.50
HARZIANIC ACID 0.5%	S+P	50	3	150	3.73±1.05	1.06 ± 0.35	4.79 ± 0.70
HARZIANIC ACID 0.1%	S	50	3	150	1.11 ± 0.10	0.58 ± 0.28	1.68± 0.18
HARZIANIC ACID 0.1%	S+P	50	3	150	9.23±1.59	4.46 ± 0.80	13.69 ± 1.99
HARZIANIC ACID 0.05%	S+P	50	3	150	2.65±0.85	4.51 ± 0.87	7.16 ± 1.64
6-PENTYL-A-PYRON 5%	S	50	3	150	0.36 ± 0.25	1.95 ± 0.68	2.32 ± 0.93
6-PENTYL-A-PYRON 5%	S+P	50	3	150	2.66 ± 0.28	3.4 ± 0.55	6.06 ± 0.28
6-PENTYL-A-PYRON 1%	S	50	3	150	0.76 ± 0.30	1.31 ± 0.43	2.08 ± 0.40
6-PENTYL-A-PYRON 1%	S+P	50	3	150	1.25 ± 0.58	0.83 ± 0.48	2.08 ± 0.54
LIPOPEPTIDES 5%	S	50	3	150	0.86 ± 0.25	2.70 ± 0.55	3.56 ± 0.32
LIPOPEPTIDES 5%	S+P	50	3	150			

LIPOPEPTIDES 1%	S	50	3	150	1.05 ± 0.22	3.63 ± 0.92	4.68 ± 1.07
LIPOPEPTIDES 1%	S+P	50	3	150			

ANCOVA results showed that the diet type was significantly influent on the average number of laid eggs per female in all the treatments with a significantly higher number in (S+P)-diet. Treatment concentration was also evidenced as statistically influent in most of the cases while the oviposition test time variable (I corresponding to the 14^{th}-17^{th} days of treatment and II corresponding to 17^{th}-20^{th} ones) was showed as influent only in some treatment.

With regard to the Control and Antibiotics treatment, the results showed very low values of R^2 and adjusted R^2 with a high value of S, meaning that the model does not fit well the data. P value for diet type was ≤ 0.05 which means that this variable statistically linked with the variability of the female fertility in control and antibiotics dataset, with a significantly higher number of eggs laid in (S+P)-diet. On the contrary, the oviposition time was discarded as a covariate with a p-value ≥ 0.05. Concentration was not included as a variable since there was no difference in concentration into the experiment program for both antibiotics and control.

The GLM generated for **Copper Oxychloride** evidenced a value of $R^2 = 37.41$ %, an adjusted $R^2 = 27.93\%$ with S = 0.48. Both diet type and concentration were considered as covariates (p-value $_{\text{diet type}} = 0.014$; p-value $_{\text{concentration}} = 0.013$) while oviposition time variable was discarded (p-value $_{\text{ov. time}} = 0.353$). The variance inflation factors of both the covariates were very low (VIF $_{\text{diet}}$ type = 1.00; VIF $_{\text{concentration}} = 1.17$), therefore diet type and concentration resulted as independent variables and the diet type was the most influent variable on females' fertility. The same pattern was reported by the GLM generated for Harzianic Acid and 6-pentyl-α-pyron.

Harzianic Acid GLM evidenced a value of $R^2 = 55.4\%$, an adjusted $R^2 = 48.35\%$ with S = 1.87. Both diet type and concentration were considered as covariates (p value $_{\text{diet type}} = 0.000$; VIF $_{\text{diet}}$ type = 1.20; p value $_{\text{concentration}} = 0.039$; VIF $_{\text{concentration}} = 1.67$) while oviposition time variable was discarded (p value $_{\text{ov. time}} = 0.053$).

6-pentyl-α-pyron GLM evidenced a value of $R^2 = 54.7\%$, an adjusted $R^2 = 47.9\%$ with S = 0.76. Both diet type and concentration were considered as covariates (p value $_{\text{diet type}} = 0.007$; VIF $_{\text{diet}}$ type = 1; p value $_{\text{concentration}} = 0.003$; VIF $_{\text{concentration}} = 1$) while oviposition time variable was discarded (p value $_{\text{ov. time}} = 0.053$). Therefore, the number of eggs laid by flies treated with Copper Oxychloride, Harzianic Acid and 6-pentyl-α-pyron is significantly lower in females fed on S-diet and treated with higher concentrations of the compound.

It was not possible to generate a GLM with oviposition test time, diet type and concentration as variables for **Viridiol** and **Lipopeptides** treatments since for these two compounds very high rates of mortality were associated with (S+P)-diet and there were no data available for both diet type at the same concentration. Thus, a second ANCOVA was carried out within the data subsets S-diet and (S+P)-diet using concentration and oviposition test time as variables.

With regards to S-diet, ANCOVA showed a high rate of variability in females' fertility linked with the treatment concentration and/or the oviposition test time. In Copper Oxychloride the treatment concentration was highlighted as a covariate with a p-value = 0.015. showing that the more concentrated is the diet fed by the flies the less they are able to produce and lay eggs regardless of the time of the oviposition test. In Harzianic Acid treatment both oviposition time and treatment concentration were evidenced as covariates ($p\text{-value}_{concentration} = 0.001$ and $p\text{-value}_{ov.\ time} = 0.001$) showing a higher number of laid eggs in the first oviposition test in comparison with the second one and an inversely proportional correlation with the treatment concentration. In Lipopeptides and 6-pentyl-α-pyron treatments, the oviposition test time was underlined as a covariate (p-value =0.000 and p-value = 0.006 respectively) with a statistically higher number of laid eggs in the second oviposition in comparison with the first one. On the contrary, the fertility of females treated with Viridiol showed to be not influenced by both the variables concentration and oviposition test time.

With regard to (S+P)-diet, the ANCOVA displayed a high rate of variability in females' fertility influenced by the oviposition test time and/or the treatment concentration. In Control and Antibiotics treatments, oviposition test was evinced as a covariate (p-value =0.043 and p-value = 0.019 respectively), with a significantly higher number of laid eggs in the first oviposition test in comparison with the second one. The same trend was presented by Copper Oxychloride treatment in which the oviposition time test was significantly influent (p-value = 0.040) while concentration was not linked with the female fertility (p-value = 0.489). In females treated with Harzianic Acid, the number of laid eggs was influenced by both treatment concentration (p-value = 0.002) and oviposition test time (p-value = 0.048), with a significantly higher number in the first oviposition test in comparison with the second one. In 6-pentyl-α-pyron treatment, the females' fertility was showed as independent from the oviposition test time (p-value = 0.635), while concentration was displayed as a covariate (p-value = 0.000), with a significantly higher number of laid eggs in the most concentrated treatment in comparison with the less concentrated one.

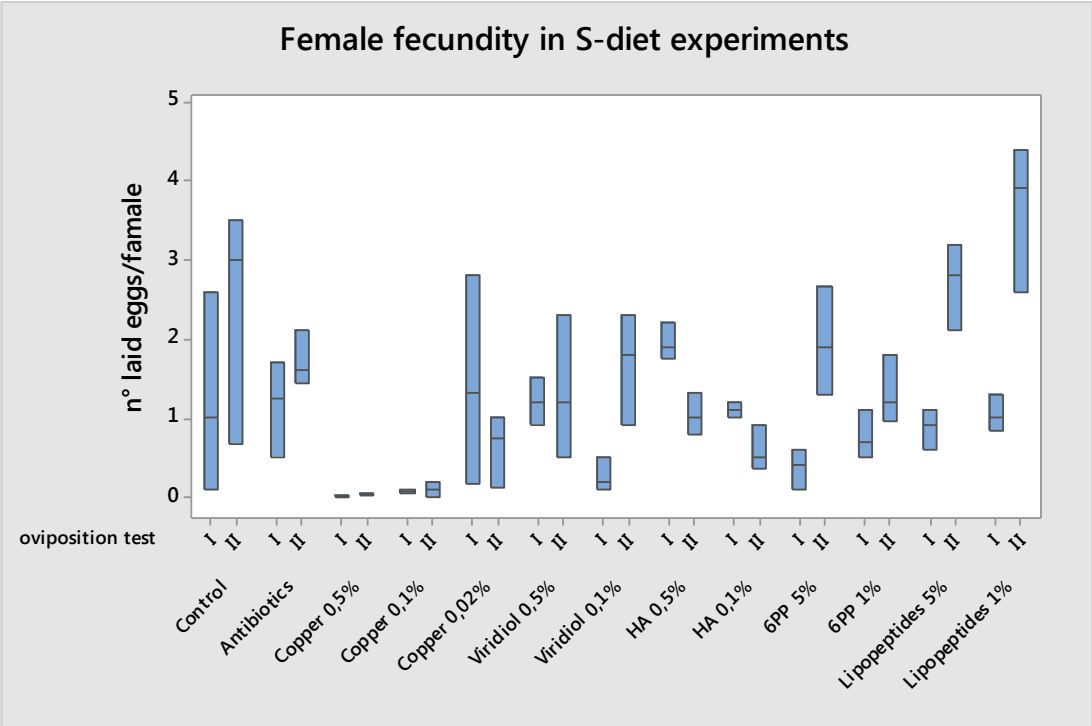

Figure 14: Boxplot of fecundity of females fed on S- diets expressed as mean number of laid eggs per female during the first and the second oviposition test

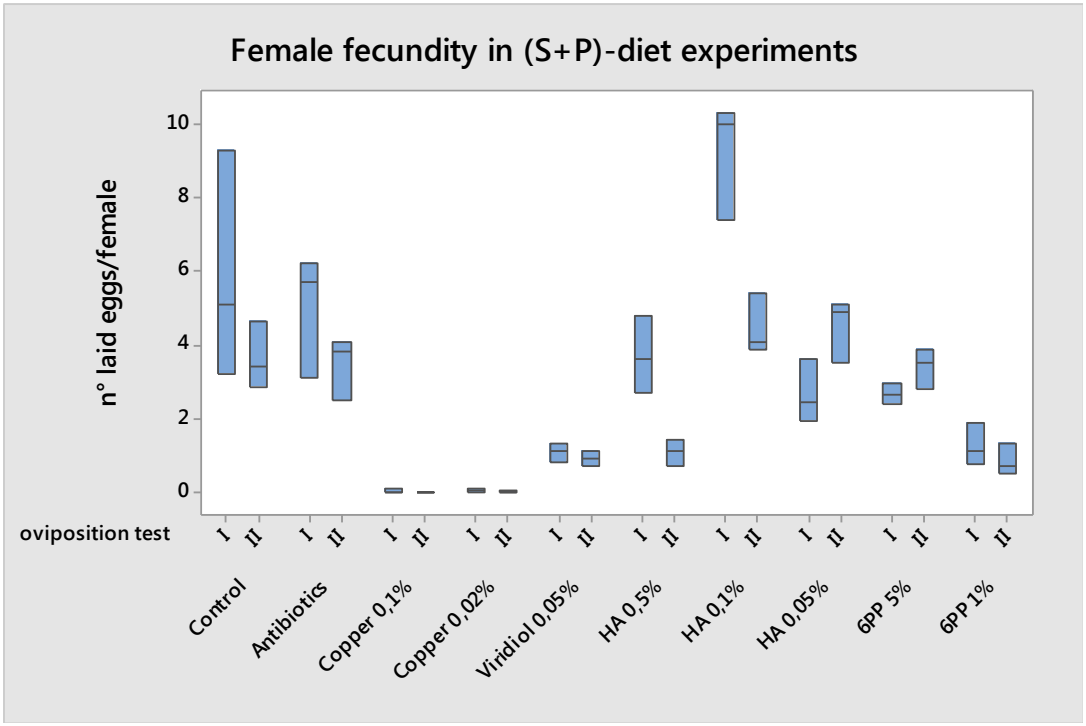

Figure 15: Boxplot of fecundity of females fed on (S+P)-diet expressed as mean number of laid eggs per female during the first and the second oviposition test

Since ANCOVA did not display a clear pattern with regard to the oviposition time test, showing that in most of the treatment this variable was irrelevant, another ANCOVA was carried out on the total number of eggs laid by females regardless of the oviposition time.

With regard to S-diet, the analysis showed that the treatment concentration was not influent on the variation of the fertility of females treated with Copper Oxychloride, Viridiol, 6-pentyl-α-pyron, and Lipopeptides, while the only treatment where the treatment concentration was evidenced as a covariate was the Harzianic Acid, with a p-value = 0.013.

With regard to (S+P)-diet, the treatment concentration was statistically influent on the number of eggs laid by females treated with Harzianic Acid and 6-pentyl-α-pyron (p-value = 0.001 and p-value = 0.00 respectively).

From the whole dataset of the total number of laid eggs (Figure 16) and carrying out another ANCOVA with diet type and treatment concentration as variables, it emerges that the diet had a high rate of influence on the female fertility, with a significant increase in the number of laid eggs in (S+P)-diet in all the treatments.

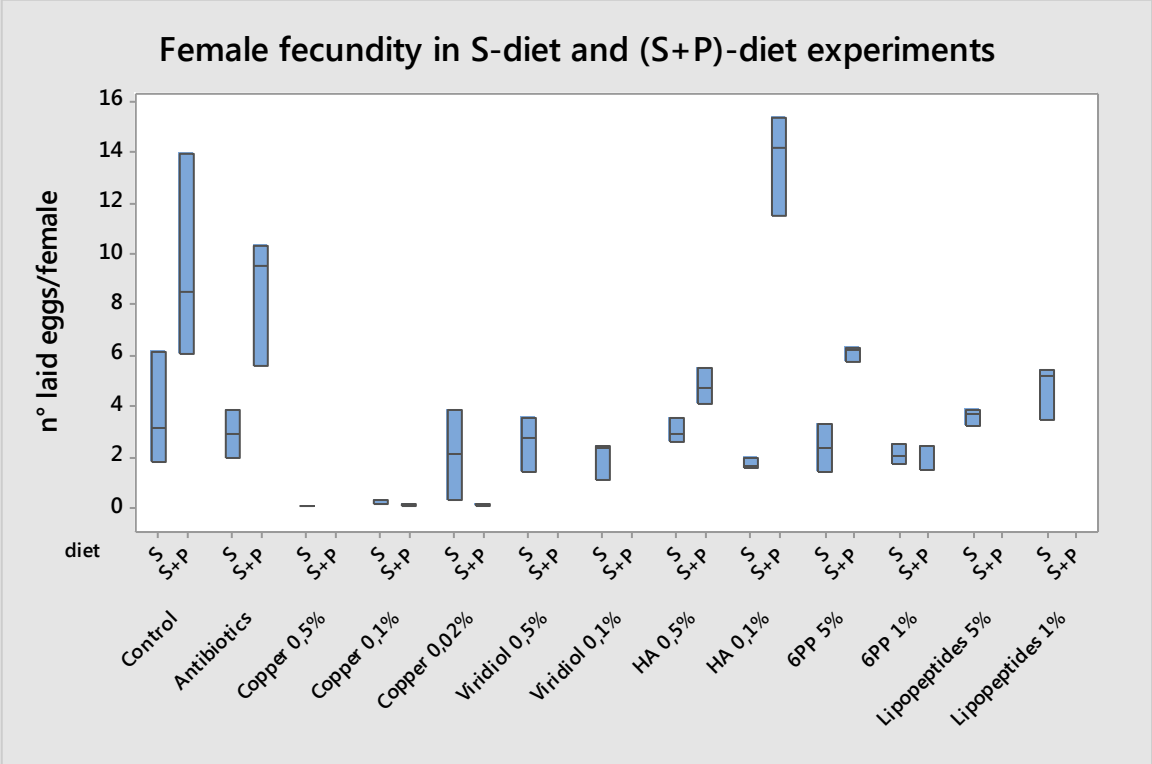

Figure 16: Boxplot of fecundity of females fed on S-diet and (S+P)-diet, expressed as total number of eggs laid per female

Furthermore, One-way ANOVA was also carried out on both diet type dataset separately to verify if the females' fertility associated with any of the treatment significantly differ from the control.

Since the oviposition time was evinced as irrelevant on the variability of the number of eggs laid in most of the treatment, ANOVA was carried out on the total number of laid eggs, recorded in the first and second oviposition tests.

With regards to S-diet, the number of eggs laid by flies treated with Copper Oxychloride at 0.5%, and 0.1% was statistically different from the Control with p values ≤ 0.05 (Figure 17).

With regard to the (S+P)-diet, the number of eggs laid by flies treated with Copper Oxychloride 0.1%, Copper Oxychloride 0.02%, Viridiol 0.05%, Harzianic Acid 0.5%, and 6-pentyl-α-pyron were statistically different from the Control one with p values ≤ 0.05. with a reduced number of laid eggs (Figure 18). Significant differences between different concentrations of the same administered compound were also recorded. In particular, Harzianic Acid 0.1% treatment was showed as statistically different from highest concentration: 0.5% (p-value = 0.000); and the lowest concentration: 0.05 (p-value =0.005), underlying that the females treated with Harzianic Acid 0.1% tend to lay a significantly higher amount of eggs in comparison with the other two treatment concentration.

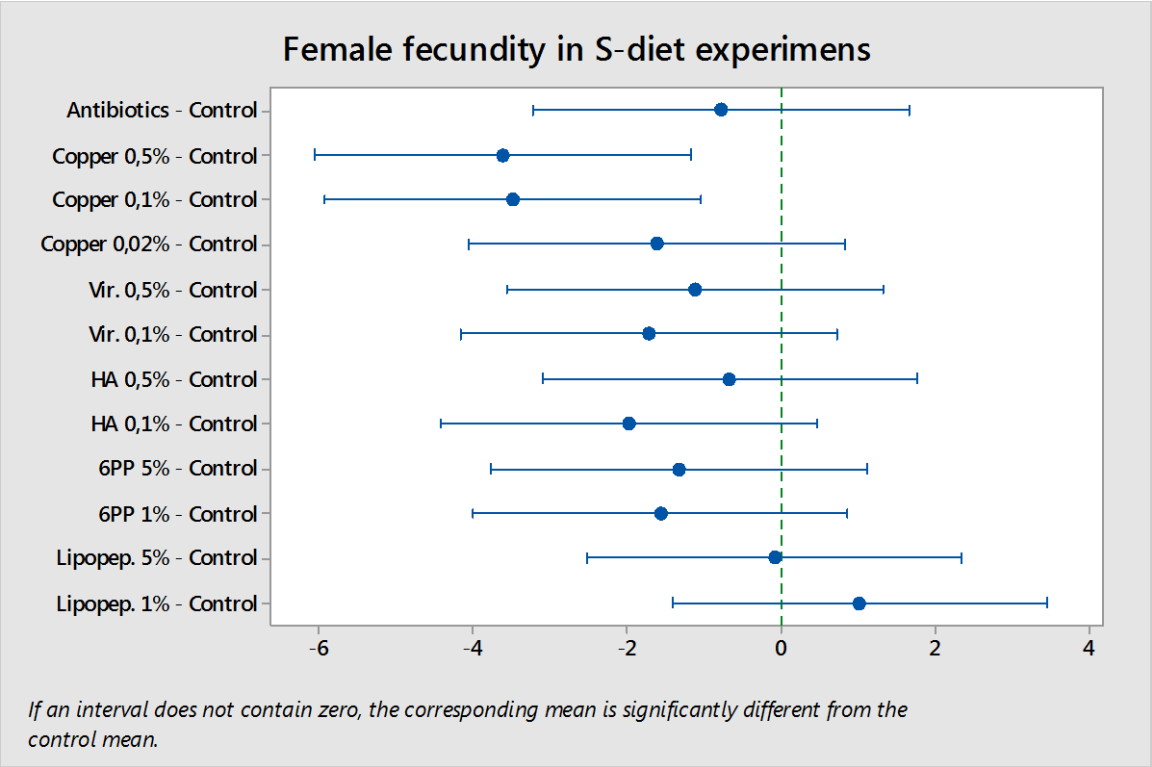

Figure 17: Dunnet test result of female fecundity in S-diet experiments

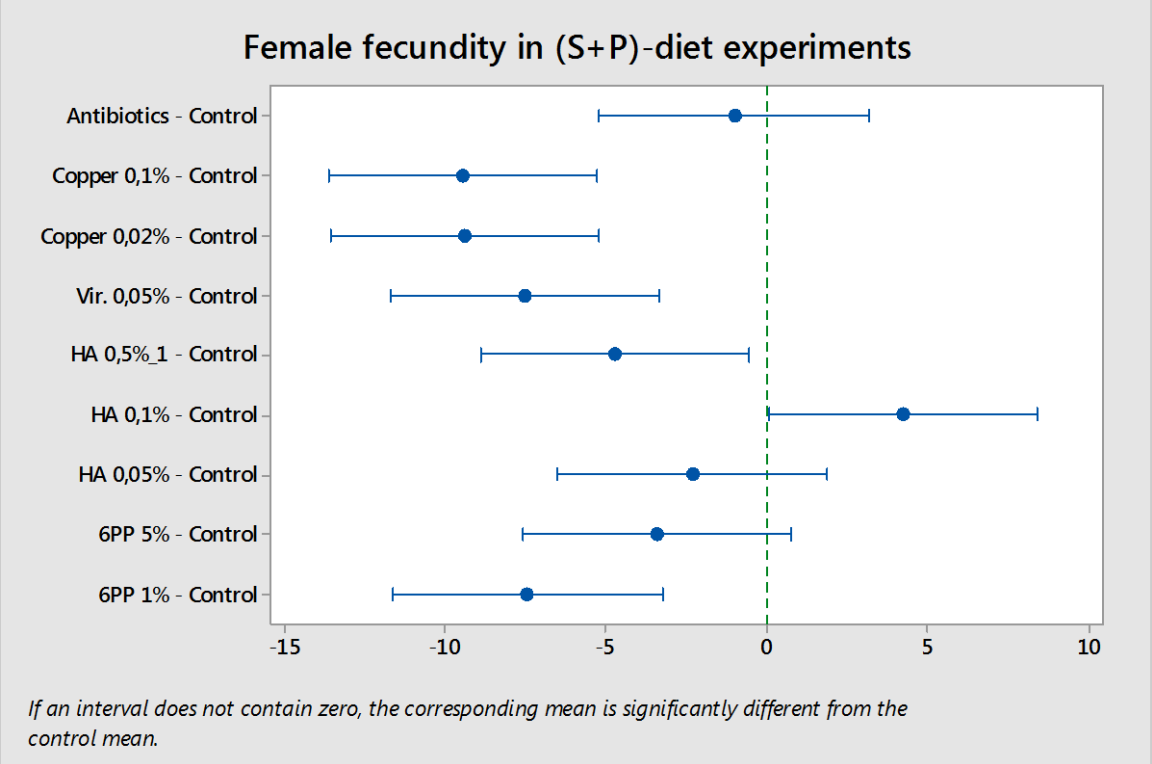

Figure 18: Dunnett test result of female fecundity in (S+P)-diet experiments

4.1.4 NUMBER OF OFFSPRING PER FEMALE

The average number of F1 offspring per female emerging from the olive fruits exposed to the treated females was analyzed to investigate the efficiency of the treatments as symbioticides. The data of some treatments on (S+P)-diet were not shown because of the high rate of mortality in one or both sexes (higher than 90%) that made impossible to carry on all the fitness test for lack of specimens. The recorded data and the summary statistic are presented in Table 7. The dataset distribution is shown in a Boxplot (Fig. 17).

Table 7: Mean number of offspring per female corresponding to each treatment ± SD in the first and second oviposition test and in total

Treatment	Diet	n° specimens/ group	n° repetitions	Total n° specimens	Mean number of offspring/female ± SD		
					I oviposition	II oviposition	total
CONTROL	S	50	5	250	0.97±0.556	0.91 ± 0.32	1.89 ± 0.64
CONTROL	S+P	50	4	200	1.20±0.29	1.31 ± 0.34	2.52 ± 0.49
ANTIBIOTICS 0.01‰	S	50	3	150	0.02±0.01	0.03 ± 0.01	0.05± 0.004
ANTIBIOTICS 0.01‰	S+P	50	3	150	0.02±0.01	0.027 ± 0.014	0.047± 0.026
COPPER OX. 0.5%	S	50	3	150	0.01 ± 0.007	0.004 ± 0.003	0.014± 0.011
COPPER OX. 0.5%	S+P	50	3	150			
COPPER OX. 0.1%	S	50	3	150	0.02 ± 0.01	0.03± 0.01	0.05± 0.02
COPPER OX. 0.1%	S+P	50	3	150	0.025±0.015	0.03 ± 0.02	0.055 ± 0.02
COPPER OX. 0.02%	S	50	3	150	0.04± 0.02	0.07 ± 0.03	0.11 ± 0.03
COPPER OX. 0.02%	S+P	50	3	150	0.08±0.02	0.09± 0.03	0.17± 0.046
VIRIDIOL 0.5%	S	50	3	150	0.50 ± 0.27	0.28 ± 0.13	0.78 ± 0.19
VIRIDIOL 0.5%	S+P	50	3	150			
VIRIDIOL 0.1%	S	50	3	150	0.20± 0.11	0.73± 0.19	0.93± 0.07
VIRIDIOL 0.1%	S+P	50	3	150			
VIRIDIOL 0.05%	S+P	50	3	150	1.04±0.39	0.32 ± 0.2	1.33 ± 0.20
HARZIANIC ACID 0.5%	S	50	3	150	0.75 ± 0.34	0.24 ± 0.08	0.99 ± 0.41
HARZIANIC ACID 0.5%	S+P	50	3	150	0.03±0.02	0.023 ± 0.01	0.053± 0.035
HARZIANIC ACID 0.1%	S	50	3	150	0.18 ± 0.13	0.02 ± 0.01	0.20 ± 0.13
HARZIANIC ACID 0.1%	S+P	50	3	150	0.04±0.02	0.033 ± 0.032	0.073 ± 0.04
HARZIANIC ACID 0.05%	S+P	50	3	150	1.03±0.3	0.15± 0.13	1.1 ± 0.17
6-PENTYL-A-PYRON 5%	S	50	3	150	0.41± 0.36	0.25 ± 0.24	0.65 ± 0.17
6-PENTYL-A-PYRON 5%	S+P	50	3	150	0.021 ± 0.02	0.009± 0.003	0.03 ± 0.022
6-PENTYL-A-PYRON 1%	S	50	3	150	0.21 ± 0.10	0.20 ± 0.11	0.42 ± 0.16
6-PENTYL-A-PYRON 1%	S+P	50	3	150	0.11± 0.08	0.14± 0.10	0.25 ± 0.18
LIPOPEPTIDES 5%	S	50	3	150	0.005 ± 0.005	0.017 ± 0.014	0.022± 0.013
LIPOPEPTIDES 5%	S+P	50	3	150			

LIPOPEPTIDES 1%	S	50	3	150	0.043 ± 0.49	0.19± 0.04	0.23 ± 0.06
LIPOPEPTIDES 1%	S+P	50	3	150			

ANCOVA results showed that oviposition test time was always irrelevant as variable thus it was excluded from the model and an additional ANCOVA was performed on the total number of offspring dataset using only diet type and treatment concentration as covariates.

Results showed that the diet type was significantly influent on the average number offspring per female in some of the treatment. Treatment concentration was evidenced as statistically influent only on Copper Oxychloride treatment.

With regards to the Control and Antibiotics treatment, the results showed very low values of R^2 and adjusted R^2 and high value of S, with the model that does not fit well the data. The GLM generated for Control dataset displayed a p-value for diet type ≤ 0.05 which means that this variable statistically linked with the variability of the number of offspring, with a significantly higher number in (S+P)-diet. On the contrary, the GLM generated for Antibiotics treatment showed that the number of offspring was not influenced by the diet type (p-value ≥ 0.05). The variable concentration was not included in the model since there was no difference among the applied concentration for both antibiotics and control treatments.

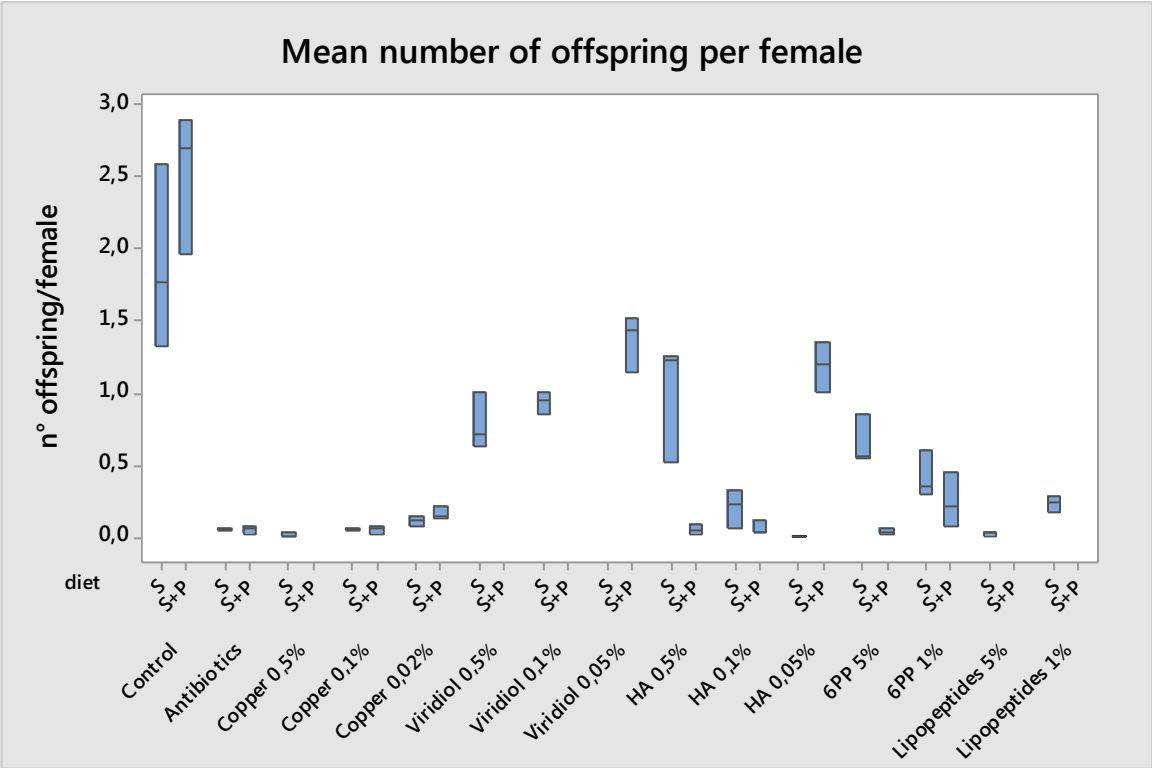

Figure 19: Mean number of offspring per female at each treatment on S-diet and (S+P)-diet

The GLM generated for **Copper Oxychloride** evidenced a value of R^2 = 76.61 %, an adjusted R^2 = 70.22% with S = 0.03. Concentration treatment was considered as covariate (p-value $_{concentration}$ = 0.001) while diet type variable was discarded (p-value $_{diet\ type}$ = 0.211;). The variance inflation factor of the covariate was very low (VIF $_{concentration}$ = 1.00), therefore diet type is an independent variable and that is strongly influent on the offspring number of females treated with Copper Oxychloride.

The GLM generated for evidenced very low values of R^2 and adjusted R^2 with a high value of S, and the model that does not fit well the data. Both diet type and concentration were discarded as covariates with p values ≥ 0.05 meaning that these variables were not linked to a number of offspring variability looking at the two diet type dataset at the same time.

The GLM generated for **6-pentyl-α-pyron** evidenced a value of R^2 = 58.7%, an adjusted R^2 = 54.9% with S = 0.19. Diet type and was considered as a covariate (p-value $_{diet\ type}$ = 0.006) while treatment concentration was discarded (p-value $_{concentration}$ = 0.934). Therefore, the number of offspring per female treated with 6-pentyl-α-pyron is highly related to the diet type, with a significantly lower amount in females fed on (S+P)-diet.

It was not possible to generate a GLM using diet type and concentration as variables for Viridiol and Lipopeptides treatments, since for both compounds a very high rate of mortality was associated with (S+P)-diet and no data were available for both diet type at the same concentration. Thus, a second ANCOVA was carried out within the data subsets S-diet and (S+P)-diet using concentration as variable.

With regards to S-diet, ANCOVA showed a high rate of variability in females' offspring number linked with the treatment concentration. In Copper Oxychloride and Lipopeptides treatments, concentration resulted as a covariate with a p-value ≤ 0.05 ($p\text{-value}_{copper} = 0.006$; $p\text{-value}_{Lipopeptides} = 0.004$), showing that the more concentrated is the treatment fed by the flies the less their offspring are able to develop and emerge. In Harzianic Acid, treatment concentration resulted as a covariate with a p-value = 0.004. But in these cases, the concentration seemed to have an opposite effect, since at higher concentration the number of recorded offspring was higher. In Viridiol and 6-pentyl-α-pyron treatments, the different concentrations did not statistically influence the offspring number.

With regard to (S+P)-diet, ANCOVA showed a high rate of variability in females' offspring number linked with the treatment concentration. In Copper Oxychloride and Harzianic Acid treatments, the concentration resulted as a covariate with a p-value ≤ 0.05 ($p\text{-value}_{Copper} = 0.025$; $p\text{-value}_{HA} = 0.000$), showing that fly emergence was inversely related with concentration. In 6-pentyl-α-pyron treatment, the concentration was statistically irrelevant on the offspring number.

Furthermore, One-way ANOVA was also performed on both diet type dataset separately to verify if the average number of offspring per female associated with any of the treatment significantly differ from the control.

With regard to S-diet , the number of offspring of females with Antibiotics, Copper Oxychloride 0.5%, Copper Oxychloride 0.1%, Copper Oxychloride 0.02%, Harzianic Acid 0.1%, 6-pentyl-α-pyron 5%, 6-pentyl-α-pyron 1%, Lipopeptides 5% and Lipopeptides 1% were evidenced as statistically different from the Control ($p \leq 0.05$), with reduced number of laid eggs in comparison with the Control (Figure 20 and Figure 21).

Significant differences among concentrations of the same administered compound were also evidenced. Copper Oxychloride 0.5% treatment resulted statistically different compared to the lowest applied concentration (0.02%), with a reduced number of offspring. The same pattern was recorded for Lipopeptides, where the highest concentration was statistically different from the

lowest one. Harzianic Acid 5% was also statistically different from the lower concentration 1%, but an opposite pattern was showed since the highest concentration led to a higher number of offspring.

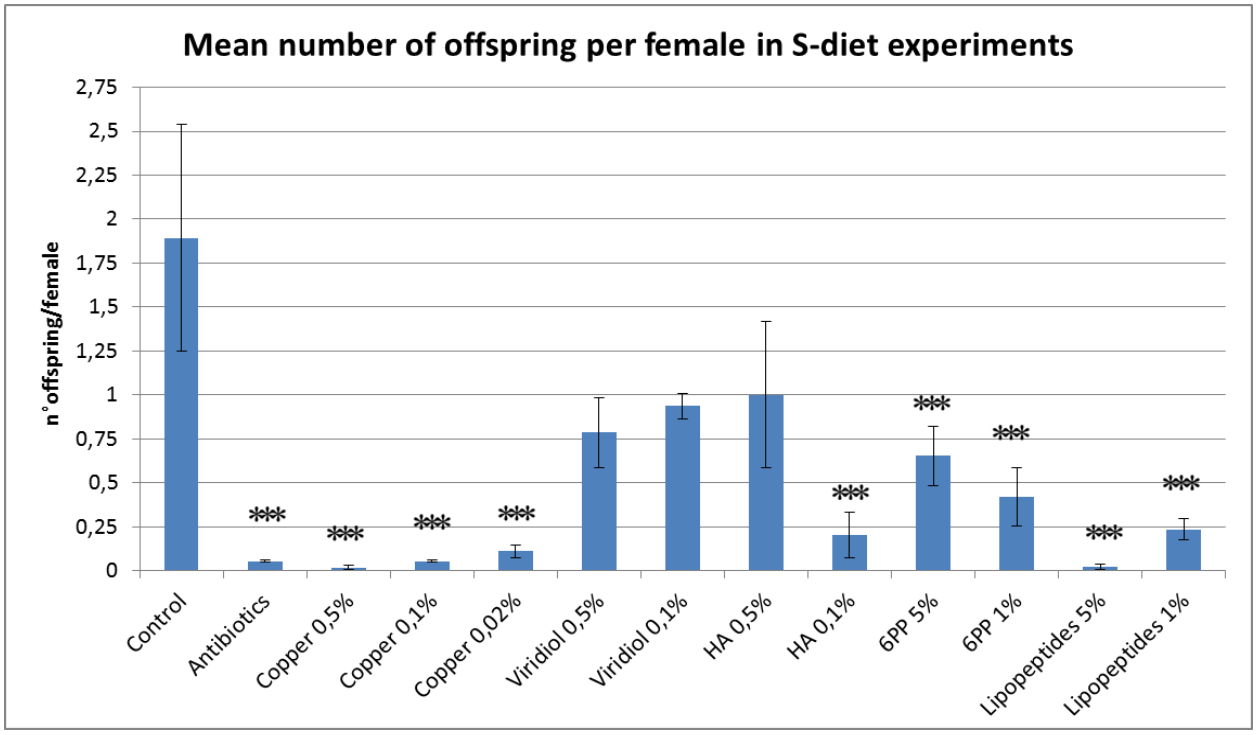

Figure 20: Mean number of offspring per female in S-diet. Bars marked with a sign (***) resulted significantly different from the control mean in the Tukey's pairwise post-hoc test performed after the ANOVA.

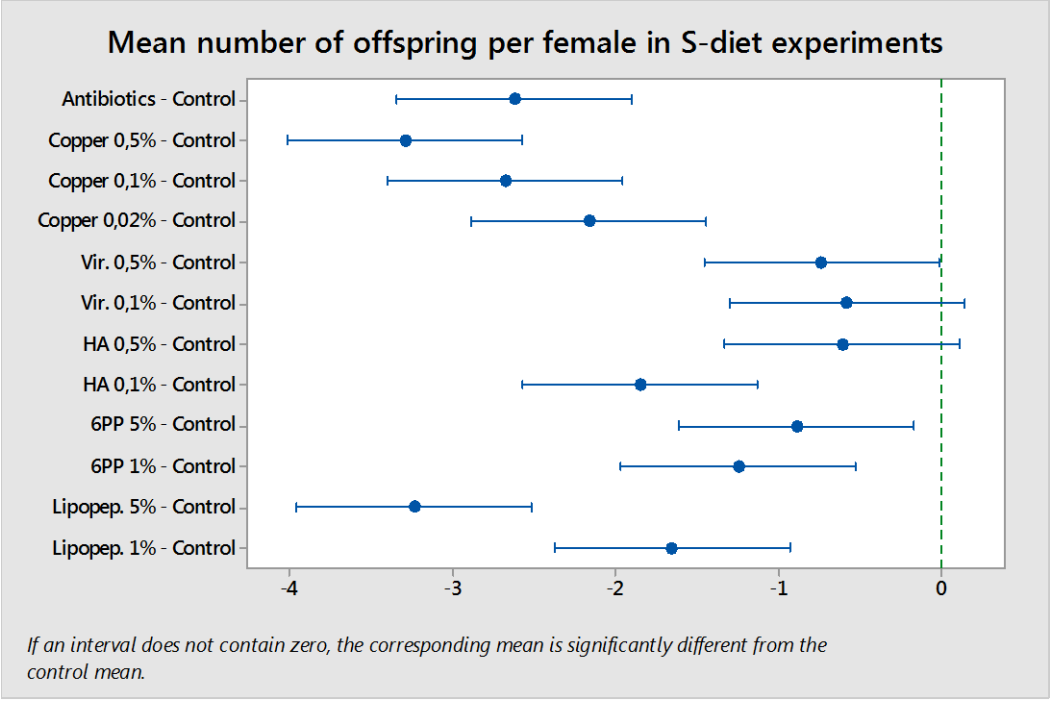

Figure 21: Dunnett test result of the mean number of offspring per female in S-diet experiments

With regard to (S+P)-diet, the number of offspring of females with Antibiotics, Copper Oxychloride 0.1%, Copper Oxychloride 0.02%, Harzianic Acid 0.5%, Harzianic Acid 0.1%, 6-pentyl-α-pyron 5% and 6-pentyl-α-pyron1 resulted statistically different from the Control (p values ≤ 0.05), with reduced number of eggs laid compared to the control cohort (Figure 22 and Figure 23).

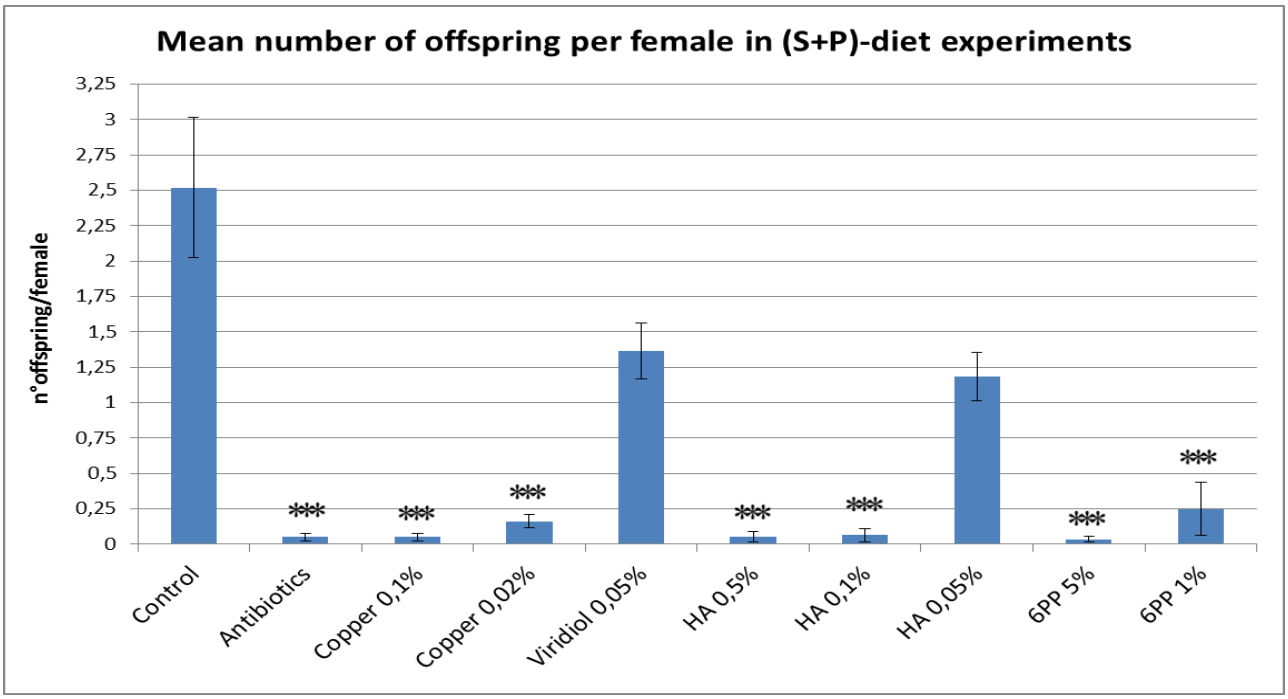

Figure 22: Mean number of offspring per female in (S+P)-diet. Bars marked with a sign (***) resulted significantly different from the control mean in the Tukey pairwise post-hoc test performed after the ANOVA.

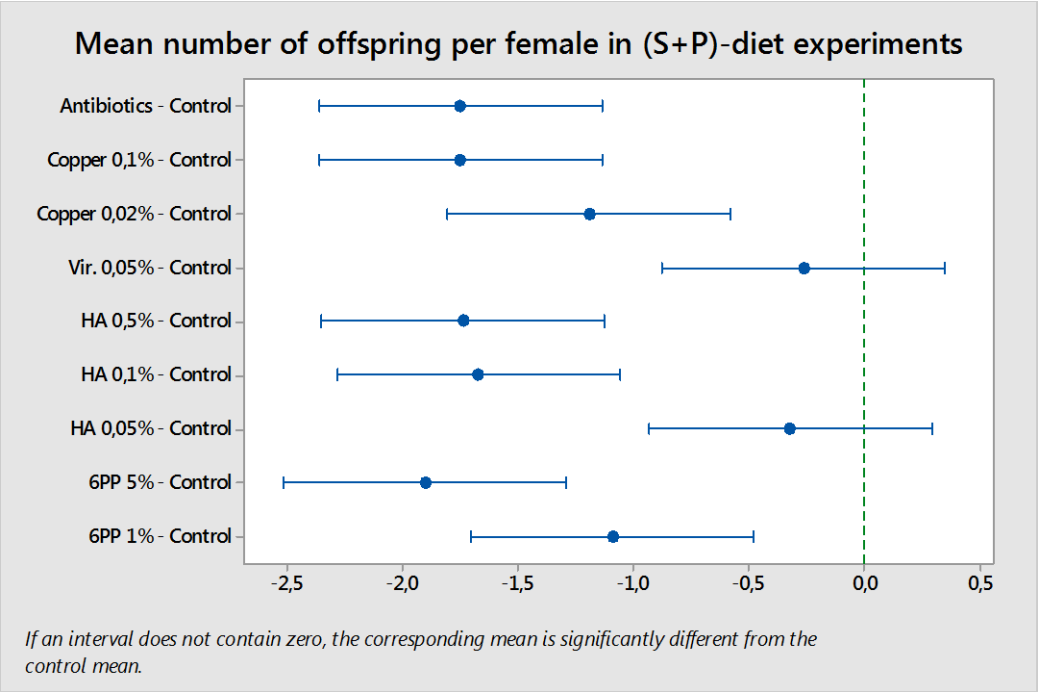

Figure 23: Dunnett test result of mean number of offspring per female in (S+P)-diet experiments

Significant differences between different concentrations of the same administered compound were also evidenced. Harzianic Acid 0.05% treatment resulted statistically different from both the higher concentration: 0.5% and 0.1% with a higher number of offspring. The same pattern was recorded in 6-pentyl-α-pyron treatments.

4.1.5 LARVAL MORTALITY

The number of laid eggs and the number of offspring per female datasets were compared to verify the larval mortality rate. The recorded data and are presented in Table 8. The comparison between the two recorded parameters is shown in two Barcharts, separately for S-diet and (S+P)-diet (Figure 24 and Figure 25).

Table 8: Rate of larval mortality recorded in each treatment. (Average offspring/female - eggs/female)

Treatment	Diet	n° eggs/female	n° offspring/female	Larval mortality
CONTROL	S	3.65	1.89	48.22%
CONTROL	S+P	9.5	2.52	73.47%
ANTIBIOTICS 0.01‰	S	2.86	0.05	98.25%
ANTIBIOTICS 0.01‰	S+P	8.47	0.047	99.45%
COPPER OX. 0.5%	S	0.04	0.014	65.00%
COPPER OX. 0.5%	S+P			

COPPER OX. 0.1%	S	0.17	0.05	70.59%
COPPER OX. 0.1%	S+P	0.044	0.055	-25.00%
COPPER OX. 0.02%	S	2.05	0.11	94.63%
COPPER OX. 0.02%	S+P	0.073	0.17	-132.88%
VIRIDIOL 0.5%	S	2.54	0.78	69.29%
VIRIDIOL 0.5%	S+P			
VIRIDIOL 0.1%	S	1.93	0.93	51.81%
VIRIDIOL 0.1%	S+P			
VIRIDIOL 0.05%	S+P	1.96	1.33	32.14%
HARZIANIC ACID 0.5%	S	2.99	0.99	66.89%
HARZIANIC ACID 0.5%	S+P	4.79	0.053	98.89%
HARZIANIC ACID 0.1%	S	1.68	0.2	88.10%
HARZIANIC ACID 0.1%	S+P	13.69	0.073	99.47%
HARZIANIC ACID 0.05%	S+P	7.16	1.1	84.64%
6-PENTYL-A-PYRON 5%	S	2.32	0.03	98.71%
6-PENTYL-A-PYRON 5%	S+P	6.06	0.03	99.50%
6-PENTYL-A-PYRON 1%	S	2.08	0.42	79.81%
6-PENTYL-A-PYRON 1%	S+P	2.08	0.25	87.98%
LIPOPEPTIDES 5%	S	3.56	0.022	99.38%
LIPOPEPTIDES 5%	S+P			
LIPOPEPTIDES 1%	S	4.68	0.23	95.09%
LIPOPEPTIDES 1%	S+P			

Larval mortality resulted very high in most of the treatment with no exception for the control. The amount of offspring in comparison with the number of laid eggs was particularly low in S-diet for Antibiotics, Copper Oxychloride 0.02%, Harzianic Acid, and Lipopeptides. In (S+P)-diet the larval mortality was particularly high in Antibiotics, Harzianic Acid, and 6-pentyl-α-pyron.

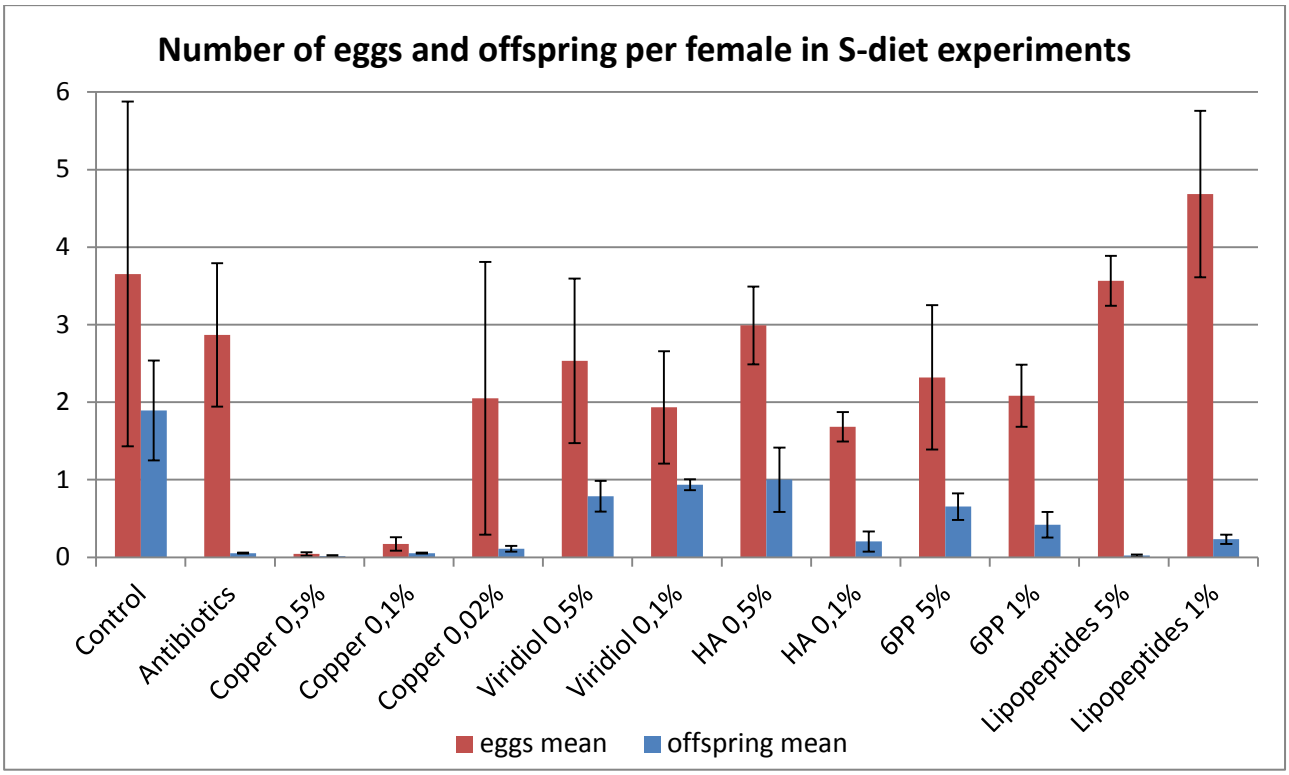

Figure 24: *Barcharts of mean number of laid eggs and mean number of offspring per female fed on S-diet*

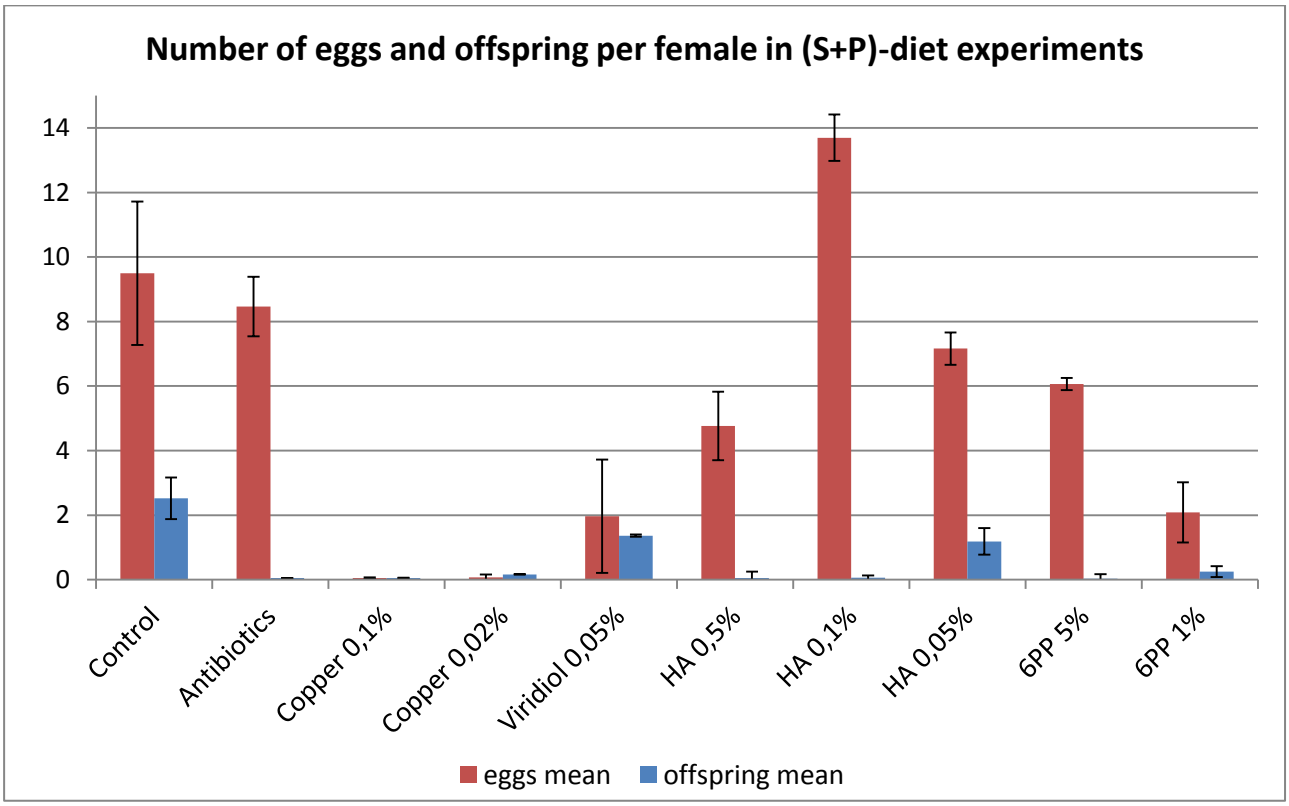

Figure 25: Barcharts of mean number of laid eggs and mean number of offspring per female fed on (S+P)-diet

4.2 ENDOSYMBIOTIC BACTERIA QUANTIFICATION BY REAL-TIME PCR

Results obtained from real-time PCR showed that most of the treatment had a negative impact on the *B. oleae* microbiome, with a significant decrease of the bacterial titer calculated relatively to the β-actin gene of the insect with the $2^{-(\Delta\Delta Ct)}$ method.

Unfortunately, a storage problem of the DNA samples of 6-Pentyl-α-Pyron and Lipopeptides made impossible to carry out the real-time PCR on these samples. All the other treatments were successfully processed and the bacterial quantification was achieved.

4.2.1 ENDOSYMBIOTIC BACTERIA QUANTIFICATION IN MID-GUT SAMPLES

With regards to the results obtained from mid-gut samples of females fed on diet S, Levene's test confirmed the homogeneity of variance from means and medians (p-value means = 0.337; p-value medians = 0.467). The normality of the residuals distribution was verified with the Shapiro-Wilk test (W=0.961; p normal = 0.064).

The ANOVA showed that the bacterial titer was significantly lower in samples treated with Antibiotics, Copper Oxychloride 0.1% and Viridiol 0.1% while in copper oxychloride 0.5% and 0.02% it was lower too, but with a p-value a little higher than 0.05 (Figure 26 and Figure 27).

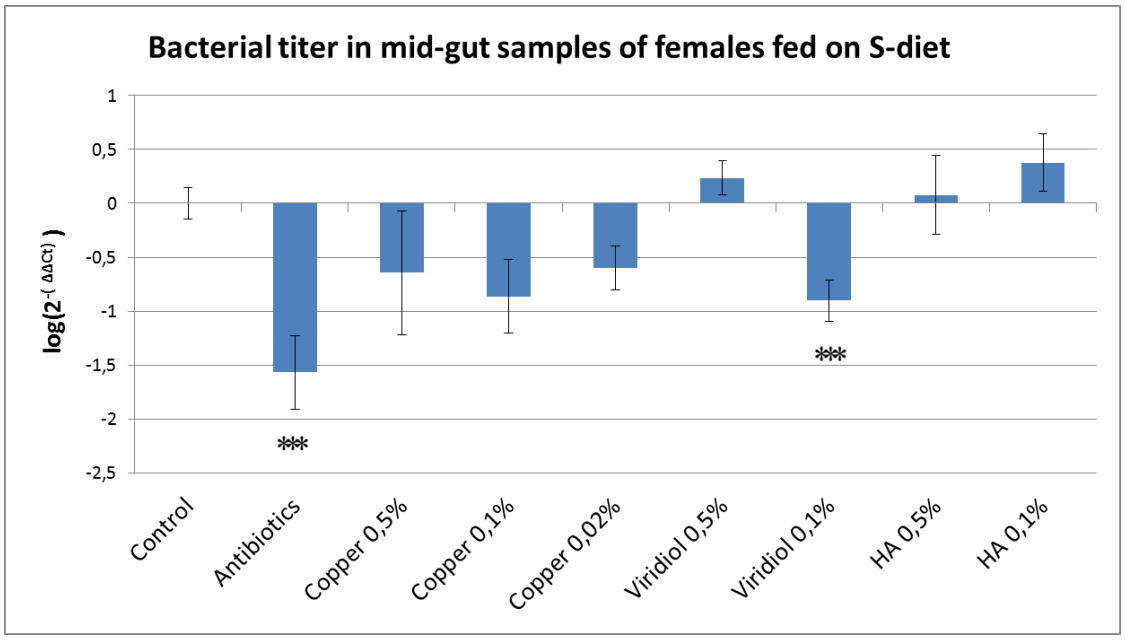

Figure 26: Bacterial titer resulted by real-time PCR and expressed as log ($2^{-(\Delta\Delta Ct)}$) in mid-gut samples of females fed on S-diet. Bars marked with a sign (***) resulted significantly different from the control mean in the Tukey pairwise post-hoc test performed after the ANOVA.

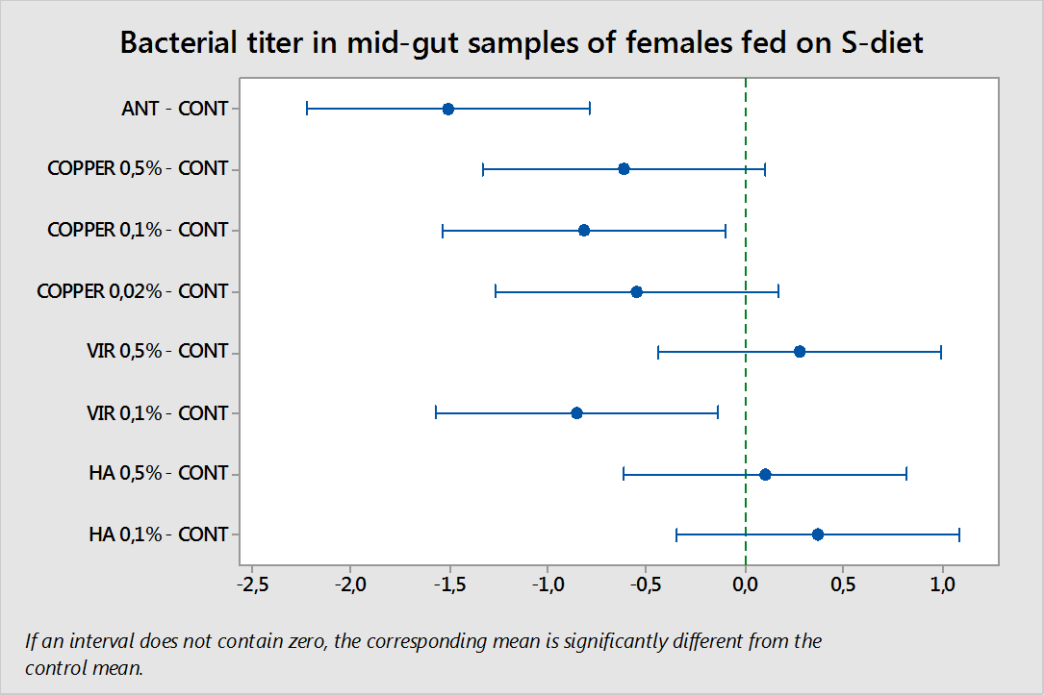

Figure 27: *Dunnett test result of the bacterial titer recorded in mid-gut samples of females fed on S-diet.*

With regards to the results obtained from mid-gut samples of females fed on diet S+P, Levene's test confirmed the homogeneity of variance from means and medians (p-value means = 0.258 p-value medians = 0.442). The normality of the residuals distribution was verified with the Shapiro-Wilk test (W=0.947; p normal = 0.052).

ANOVA showed that the bacterial titer was significantly lower only in samples treated with Antibiotics while in Copper Oxychloride 0.02% it resulted to be significantly higher (Figure 28 and Figure 29).

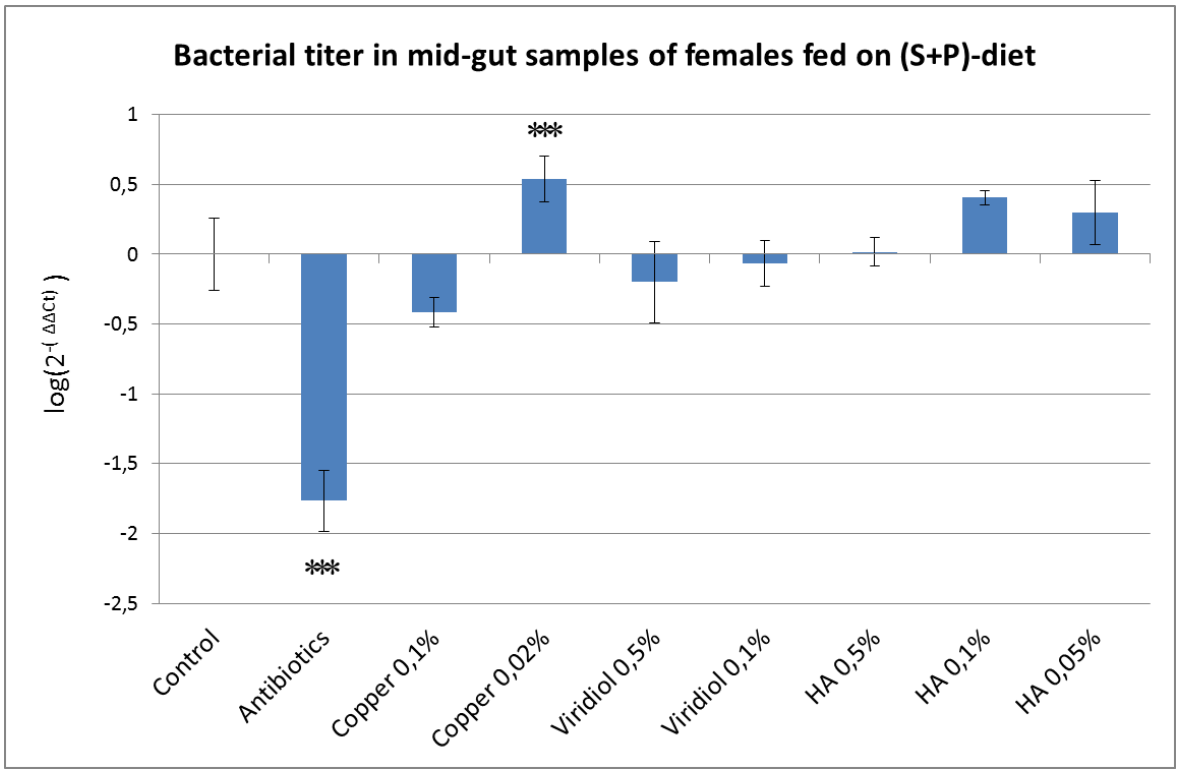

Figure 28: Bacterial titer resulted by real-time PCR and expressed as log (2-(ΔΔCt)) in mid-gut samples of females fed on (S+P)-diet. Bars marked with a sign (***) resulted significantly different from the control mean in the Tukey pairwise post-hoc test performed after the ANOVA.

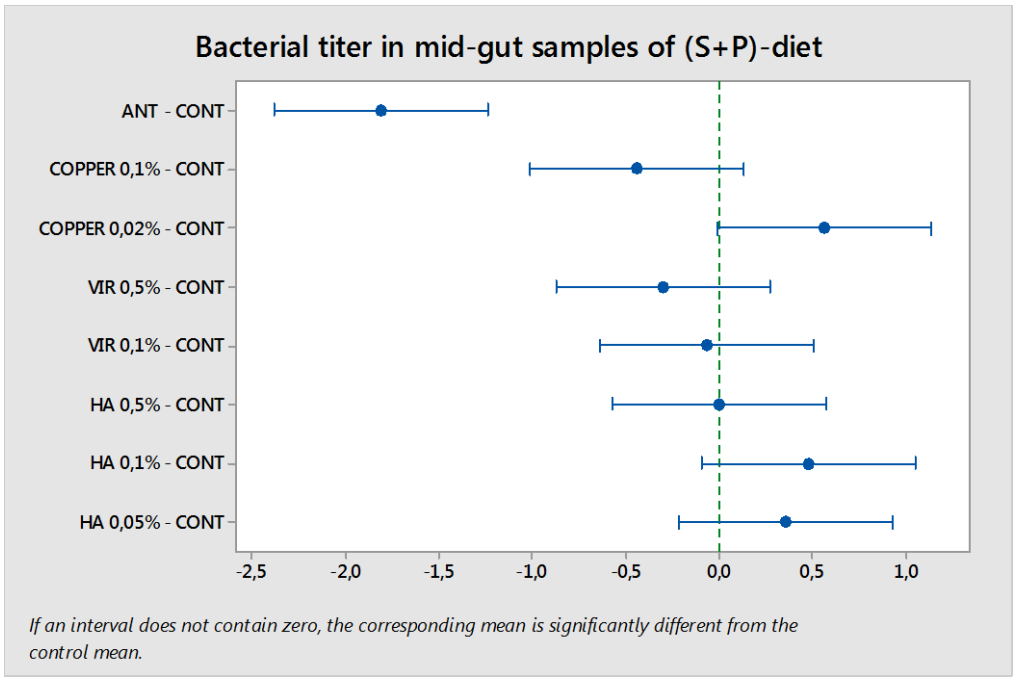

Figure 29: Dunnett test of the bacterial titer recorded in mid-gut samples of females fed on (S+P)-diet

4.2.2 ENDOSYMBIOTIC BACTERIA QUANTIFICATION IN OESOPHAGEAL BULB SAMPLES

With regard to the results obtained from oesophageal bulb samples of females fed on diet S, Levene's test confirmed the homogeneity of variance from means and medians (p-value means = 0.332 p-value medians = 0.524). The normality of the residuals distribution was verified with the Shapiro-Wilk test (W=0.965; p normal = 0.072).

ANOVA showed that the bacterial titer resulted to be significantly lower in samples treated with Antibiotics, Copper Oxychloride 0.02%, Viridiol 0.5%, Viridiol 0.1%, Harzianic Acid 0.5% and Harzianic Acid 0.1% (Figure 30 and Figure 31).

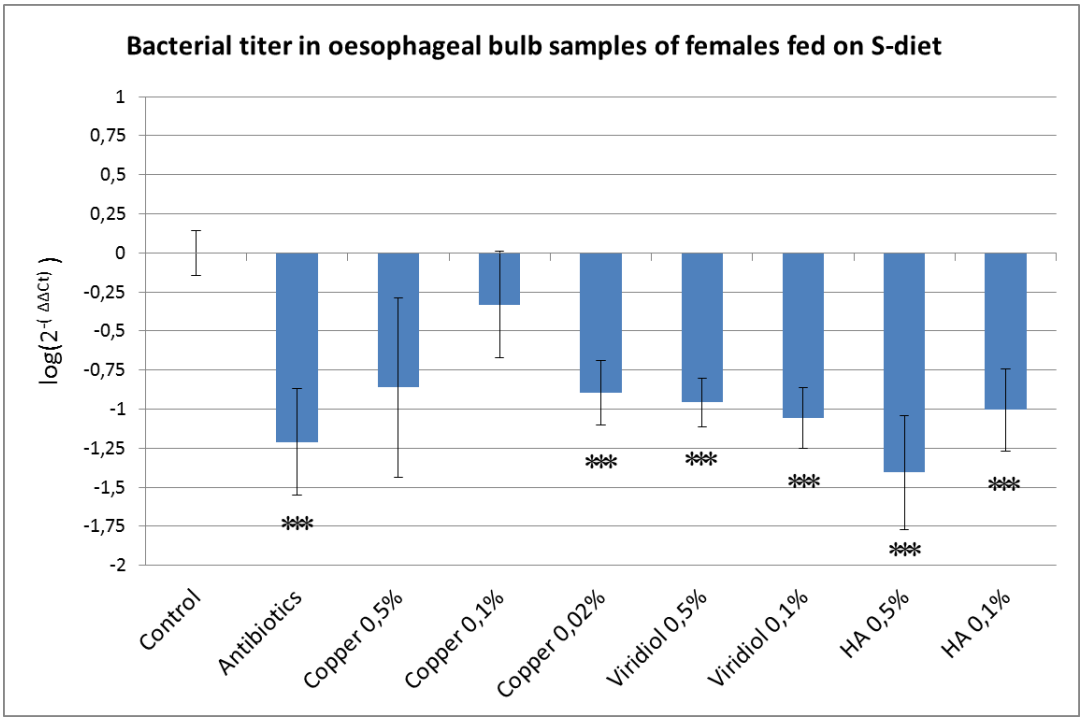

Figure 30: Bacterial titer resulted by real-time PCR and expressed as log ($2^{-(\Delta\Delta Ct)}$) in oesophageal bulb samples of females fed on S-diet. Bars marked with a sign (***) resulted significantly different from the control mean in the Tukey pairwise post-hoc test performed after the ANOVA.

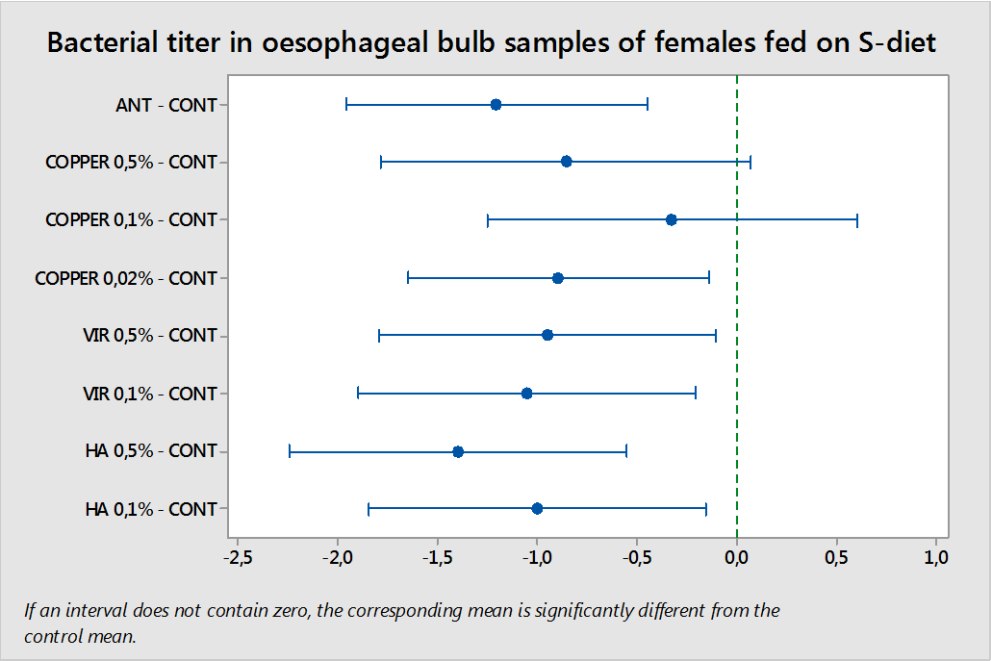

Figure 31: *Dunnett test result of the bacterial titer reported in oesophageal bulb samples of females fed on S-diet.*

With regard to the results obtained from oesophageal bulb samples of females fed on diet S+P, Levene's test confirmed the homogeneity of variance from means and medians (p-value means = 0.819 p-value medians = 0.580). The normality of the residuals distribution was verified with the Shapiro-Wilk test (W=0.975; p normal = 0.580).

ANOVA showed that the bacterial titer resulted to be significantly lower in samples treated with Antibiotics, Copper Oxychloride 0.02%, Viridiol 0.5%, Viridiol 0.1%, Harzianic Acid 0.5% and Harzianic Acid 0.1% and Harzianic Acid 0.05% (Figure 32 and Figure 33).

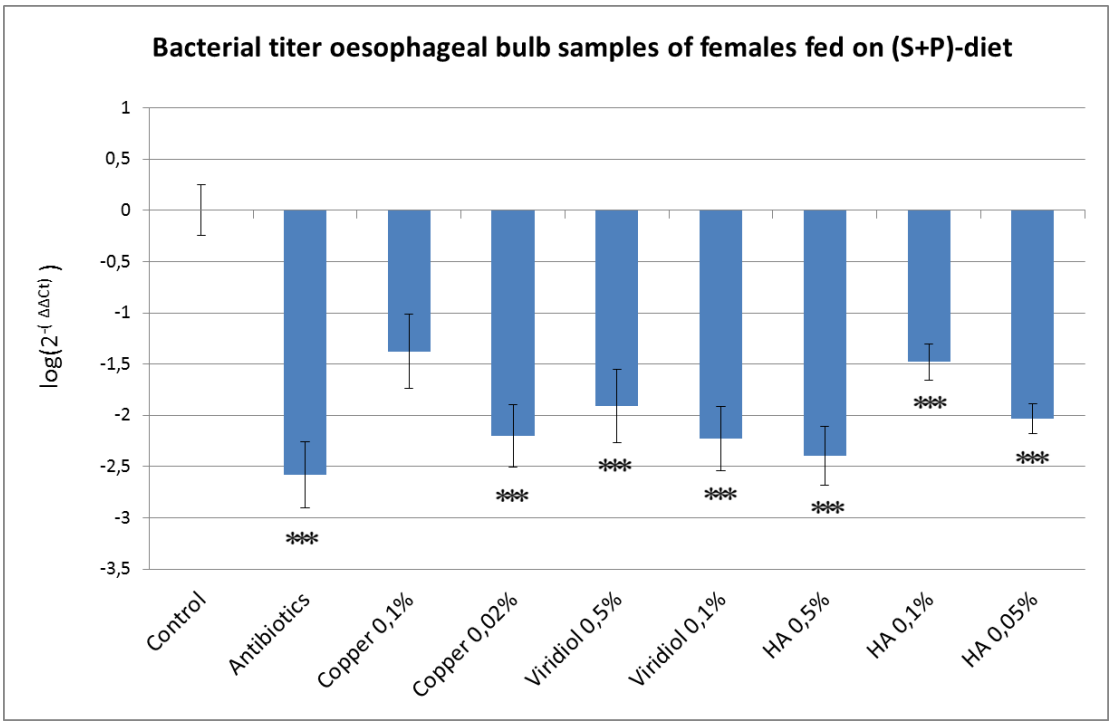

Figure 32: *Bacterial titer resulted by real-time PCR and expressed as log ($2^{-(\Delta\Delta Ct)}$) in oesophageal bulb samples of females fed on (S+P)-diet. Bars marked with a sign (***) resulted significantly different from the control mean in the Tukey pairwise post-hoc test performed after the ANOVA*

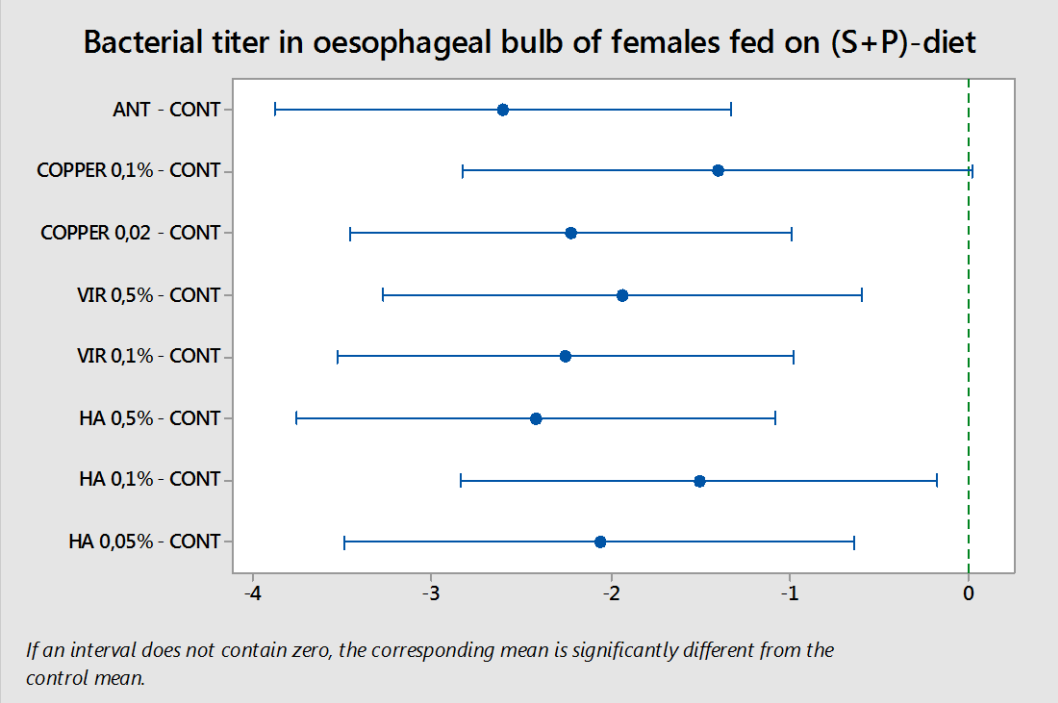

Figure 33: *Dunnett test result of the bacterial titer recorded in oesophageal bulb samples of females fed on (S+P)-diet.*

The results of ANCOVA carried out with diet type, treatment concentration, and organ type as variables, showed that the organ type was statistically influent on all the treatments with an exception for the Antibiotics, while diet treatment concentration was influent only on Viridiol and Harzianic Acid and type resulted to be linked to the bacterial titer variability only in Antibiotics treatment.

The GLM generated for **Copper Oxychloride** evidenced a value of R^2 = 27.71 %, an adjusted R^2 = 24.23% and S = 0.81. With the model that does not fit well the dataset since only ≈ 25% of the variability was explained. Organ type was considered as a covariate (p-value $_{\text{organ type}}$= 0.001) while diet type and treatment concentration variables were discarded (p-value $_{\text{diet type}}$ = 0.745; p-value $_{\text{concentration}}$ = 0.968). Therefore, the bacterial titer in flies treated with copper depends on the organ type analyzed but is independent of the diet type and the treatment concentration.

The GLM generated for **6-pentyl-α-pyron** evidenced a value of R^2 = 60.3%, an adjusted R^2 = 57.1% and with S = 0.59. Both organ type and concentration were considered as covariates (p value $_{\text{organ type}}$ = 0.000; p value $_{\text{concentration}}$ = 0.032) while diet type variable was discarded (p value $_{\text{concentration}}$= 0.053). Therefore, the bacterial titer of flies treated with **Harzianic Acid** depends on the organ type and the treatment concentration. The same pattern was evinced for **Viridiol** (p value $_{\text{organ type}}$ = 0.000; p value $_{\text{concentration}}$ = 0.045; p value $_{\text{diet type}}$= 0.143).

Performing an ANCOVA on the two organs dataset separately using diet type and treatment concentration as variables, a strong influence of the diet type was evidenced in most of the treatments. While in oesophageal bulb the presence of proteins resulted to significantly increase the reduction of the bacterial titer in all the treatments, in the mid-gut dataset the pattern displayed was not clear with a decrease of the reduction in Copper treatments and Viridiol 0.1% and no significant difference in the other treatments (Figure 34).

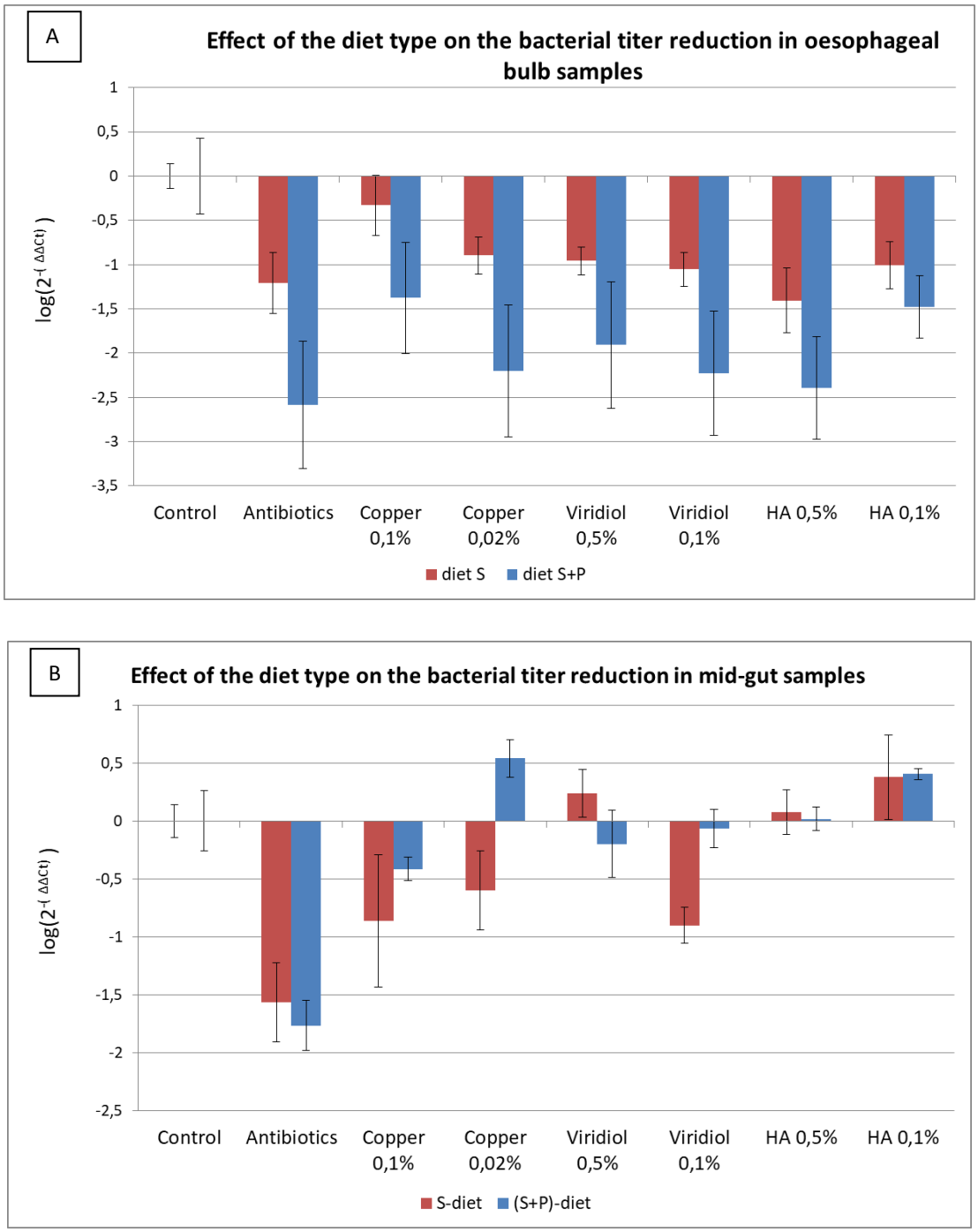

Figure 34: *Comparison among the bacterial titer resulted by the Real-Time PCR analysis in the oesophageal bulb samples (A) and mid-gut samples (B) extracted by females fed on diet S and on (S+P)-diet*

5. DISCUSSION

5.1 ADULT AND LARVAL FITNESS TESTS

5.1.1 MORTALITY

The statistical analysis carried out on mortality dataset demonstrated that the presence of protein in the diet significantly increase the mortality rate in all the treatments, except for Control and Antibiotics. The fact that the mortality rate associated with Control and Antibiotics treatment was not statistically different in both diet type experiments confirm that the lack of beneficial bacteria does not affect the survival of the adult stage (Ben-Yosef et al 2010; 2014).

Therefore, it is possible that the differences between the mortality means of control groups and treatments were not due to the microbiome alteration but it was linked with a possible toxic effect of the treatments.

ANOVA did not detect any significant difference between the means of the control groups and the treatments in the S-diet dataset (Figure 4), while in the (S+P)-diet dataset many treatments significantly differ from the control with a higher mortality rate for both sexes (Figure 5, Figure 7 and Figure 8). This is the case of Copper Oxychloride treatments with higher concentrations, 6-pentyl-α-pyron at the highest concentration and Lipopeptides at the highest concentration.

Thus, it seems that the proteins assimilated by flies somehow intensify the toxicity of the treatments. It could be hypothesized that the high-protein diet led to a metabolism acceleration that could increase the quantity of treated diet assimilated by flies, thus lowering the tolerance threshold for the administered compounds. A similar effect was already observed in a study on the influence of the diet composition associated with resistance mechanism to *Bacillus thuringiensis* in the cabbage looper (Shikano and Cory, 2014). Another study also underlined the detrimental effect of a high-protein diet in the tolerance of the burying beetle, *Nicrophorus vespilloides* to the entomopathogenic bacteria, *Photorhabdus luminescens* (Abdullah *et al.*, 2017).

A clear pattern was evidenced in Copper Oxychloride treatments where the most concentrated treatment showed a higher mortality rate than the medium and less concentrated ones (Figure 4 and Figure 6).

In Viridiol treatments administered in (S+P)-diet the mortality rate recorded in females was substantially similar to the control one, while males cohorts showed significantly higher values, up to 100% in the more concentrated treatments. Thus, data show that this particular compound is highly toxic and even lethal for male olive flies in a concentration-dependent manner.

Differential sex-linked mortality was recorded, with a significantly higher mortality in males than in females. Higher male mortality was recorded also always in more concentrated treatments, highlighting again the key role of this parameter in the toxic effect on adult files. Treated males resulted more susceptible than females in some of the treatments. These results cannot be explained by the current level of knowledge. Further analysis of the proteomic statement of the available data may clarify the functional and metabolic differences existing between males and females specimens of *B. oleae* that could explain the different responses to the same treatment.

Copper Oxychloride 0.02%, Harzianic Acid 0.1 and 0.05% and 6-pentyl-α-pyron 1% treatments, showed no toxic effect on *B. oleae* adults.

5.1.2 DAILY DIET CONSUMPTION

Two different patterns emerged by the statistical analysis of the amount of ingested diet per specimen and per day depending on the diet type.

The results obtained from the S-diet dataset showed that the daily diet intake was substantially higher in females than in males in most of the treatment including Control and Antibiotics. In fact, female flies naturally have the tendency to feed more, because of the nutritional needs required for oogenesis, as described also in the following paragraph (§ 5.1.3). It was also highlighted that the daily diet consumption of flies of both sexes diminished with the increase of treatment concentration.

This pattern was particularly clear in S-diet dataset as, for all the administered compounds, (Figure 9). Therefore, a possible explanation is that all compounds act as a repellent when administered in higher concentration even if the repellent effect was variable for each compound.

On the contrary, in (S+P)-diet dataset, the sex of the flies seems to be a less important factor, in fact, neither Control nor Antibiotics treatments put in evidence significant differences between

the amount of ingested diet by females and males. The observed difference between the two diet type datasets could be explained by the fact that in S-diet the deprivation of proteins, strategic for egg maturation led the females to feed more, trying to compensate the lack of proteins. This does not occur when the females are fed on (S+P)-diet, which is provided with all the essential amino acids.

However, the pattern that emerged from the (S+P)-dataset is less clear since, if fly sex is irrelevant on the average amount of ingested diet in the Control and Antibiotics treatment, it is still a covariate in Copper Oxychloride., Viridiol and Lipopeptides. For all these treatments, in fact, females ingest significantly more diet than males. Moreover, the treatment concentration resulted as a covariate of ingested diet in most of the treatment, however without a clear pattern (Figure 12 and Figure 13).

5.1.3 FECUNDITY

The statistical analysis carried out on the mean number of eggs laid per female confirmed that the presence of protein in the flies' diet significantly increase the fecundity of both aposymbiotic and symbiotic females, respectively treated with Antibiotics and the untreated Control group. Obviously, high-protein diets enhance females' nutrition and oogenesis regardless of the presence of endosymbiotic bacteria as reported from Ben-Yosef *et al.* (2014).

The same trend was observed for Harzianic Acid treatments where the females fed on (S+P)-diet showed an increase of the number of laid eggs between 60% and 700% in comparison with the S-diet cohorts. Females treated with the most concentrated treatment of 6-pentyl-α-pyron also evidenced a significantly higher fertility when fed on (S+P)-diet, with an increase of the number of laid eggs of 160%.

Lipopeptides, Viridiol, and Copper Oxychloride 0.5% treatments, it was impossible to verify the effect of the proteins on females' fecundity due to the high rate of mortality, associated with the (S+P)-diet.

On the contrary, females treated with Copper Oxychloride showed an opposite trend. In fact, it was observed a strong decrease in the number of laid eggs with values ranged between 75% and 96% when the treatment was administered in (S+P)-diet. Thus, the presence of proteins combined with copper led to a substantially lower females' fertility. Copper Oxychloride was

previously evidenced as probably toxic, with a higher mortality rate compared with the Control, and repellent since flies tend to avoid feeding on a diet that contains it. Therefore, it is not surprising that also the female fecundity is affected by the combination of Copper with proteins even if the mechanism behind this evidence is far from being explained. Further studies should be carried out to clarify the physiological and metabolic processes triggered by the interaction between proteins and copper. Copper compounds were already evidenced as moderately toxic when orally administered to insects in both adult and larval stages in a concentration-dependent manner (Jensen and Trumble, 2003; Cheruiyot 2012; Cheruiyot *et al.*, 2013). The fact that the toxicity seems to be increased by the high-protein diet could be again compared to the tolerance decrease observed in other studies (Shikano and Cory, 2014; Abdullah *et al.*, 2017).

Flies fed on S-diet required more time to mature eggs after mating and the number of eggs laid in the first oviposition test was lower than in the second one. An opposite trend was recorded for flies fed on (S+P)-diet, where females tend to lay more eggs in the first oviposition test than in the second one. So, it can be concluded that the high-protein diet accelerates the oogenesis.

Treatment concentration resulted to affect females' fertility in an inversely proportional manner

While in S-diet dataset, the number of eggs laid per female was significantly lower only in the most concentrated treatment of Copper Oxychloride, in (S+P)-diet dataset, most of the treatments produced a significant reduction of females fertility compared with the control cohorts (Figure 17 and Figure 18). Interestingly, a significant increase in the number of laid eggs was recorded in Harzianic Acid 1%.

The fact that the high-protein diet is often associated with a decrease in the fertility of the females should be explained again by an increase of the toxic effect caused by the treatments (dose-response).

Since the presence of endosymbiotic bacteria has been demonstrated not to be linked with the female fertility, we can assume that the differences between treatments and control should be explained by treatment's toxicity.

The treatments with Harzianic Acid 0.1 and 0.05%, 6-pentyl-α-pyron 5%, and Control, showed comparable female fertility values in both the diet type experiments thus we can affirm that these treatments do not affect the fitness of *B. oleae* females.

In any case, assuming that every compound may turn out to be toxic above a certain threshold, it is probably that most of the compounds will negatively affect the fertility of females only when combined with the high-protein diet with subsequent increase of the compounds assumption. Therefore, it could be possible that the same treatment at the same concentration could lead to different physiological response depending on the nutritional condition of the flies (Shikano and Cory, 2014; Abdullah *et al.*, 2017).

5.1.4 NUMBER OF OFFSPRING PER FEMALE

The number of F1 offspring obtained by the oviposition tests at each treatment was the most important parameter analyzed in the fitness tests. In fact, it has been demonstrated that the presence of "*Ca.* Erwinia dacicola" is essential for *B. oleae* larval survival in unripe olive fruit (Ben-Yosef *et al.*, 2015). The present study hypothesized that a substantial reduction of the offspring of the treated flies in comparison with the control was an evidence of the symbioticides effect of the treatment.

The presence of proteins in the flies' diet resulted again significant, but the influence of the diet type displayed two opposite patterns. In control groups, the presence of proteins significantly increase the number of offspring per female, but in some of the treatments such as Harzianic Acid and 6-pentyl-α-pyron, the effect was opposite with a substantial decrease. On the contrary, in Copper Oxychloride treatments (0.1% and 0.02%) no significant differences were evidenced comparing the S-diet and the (S+P)-diet dataset.

The effect of treatment concentration on the number of offspring per female produced an inversely proportional function. In Copper Oxychloride and Lipopeptides treatments, the more concentrated was the administered treatment the less numerous was the females' offspring. For all other treatments, even where a statistically significant difference was detected among the means of different concentrations of the same compound, it was not possible to establish a clear pattern.

Anyhow, results showed that most of the administered treatments led to a significant reduction of the number of offspring (Figure 20 and Figure 22).

Antibiotics treatment did not affect the fecundity of *B. oleae* treated females with levels comparable with their symbiotic counterparts. However, antibiotic treatment led to a reduction in

the number of offspring up to the 98% compared with the control cohort. The data confirm that the absence of endosymbiotic bacteria in mothers caused the incapability of the offspring larvae to successfully develop in unripe olive fruit (Ben-Yosef *et al.*, 2015).

Therefore, looking for potentially symbioticides compounds, active ingredients that showed to have an effect on the flies' fitness similar to the Antibiotics, have to be taken into accounts.

5.1.5 LARVAL MORTALITY

Larval mortality resulted very high in most of the treatment with no exception for the control, and in particular, for control flies fed on (S+P)-diet. In this condition, females were able to lay almost 10 eggs per olive fruit but only about 2.5 could successfully develop and reach the adult stage. That could be explained by the fact that the olive fruits exposed into the cages faced with a progressive drying and maybe it was impossible in this kind of fruits to host more than 2.5 larvae and support their development.

Some treatment resulted to have negative larval mortality (Table 8). In these cases, the number of laid eggs per olive fruit was very low with less than 0.01 eggs/olive. That could lead to an error of estimation of the egg number per olive since the number is so low and it is possible that just 1 egg was not detected during the dissection or that the number of eggs into the two groups of olive fruit wasn't identical, that would explain these abnormal results.

In any case, the larval mortality computation seemed to be scarcely important in those treatments where the number of laid eggs was less than 0.1 eggs/female.

On the contrary, interesting results were found in some treatment where although the number of laid eggs was high, the number of offspring developed from those eggs was very low (Figure 24 and Figure 25). This is the case of Harzianic Acid, 6-pentyl-α-pyron, and Lipopeptides.

5.1.6 SUMMARY OF THE FITNESS RESPONSE TO EACH COMPOUND

The effects of the evaluated compounds on the fitness of the olive fly are summarized herein:

- Copper Oxychloride administering led to a significant decrease of the number of eggs laid in at highest concentration in both the diet type. The number of offspring per female was significantly lower in all concentrations for both diet types. The presence of protein reduces the olive fly tolerance threshold to this compound. The same trend was observed for the mortality rate, not significantly different from the control in S-diet but higher in (S+P)-diet for two treatments (0.5% and 0.1%). The quantity of ingested diet was significantly lower in S-diet at 0.5% concentration and in (S+P)-diet at 0.1%. This compound has a moderate to high toxic effect on the fly fitness depending on the treatment concentration and on the presence of protein in the diet. Even if toxic effects can be expected, also a symbioticides effect has been recorded. Therefore, a combination of toxic, repellent and symbioticide effects could be the most likely hypothesis. Copper Oxychloride was already evidenced as moderately toxic when orally administered to insects in both adult and larval stages in a concentration-dependent manner (Jensen and Trumble, 2003; Cheruiyot 2012; Cheruiyot *et al.*, 2013). Anyhow, these results would confirm the previous study about the possible symbioticide effect of Copper on olive fruit fly endosymbiont (Rosi *et al.*, 2007; Sacchetti *et al.*, 2004; Belcari and Bobbio, 1999; Tzanakakis, 1985).

- Viridiol led to a significantly higher mortality of males when the compound was administered with the addition of proteins. The high mortality of males in (S+P)-diet did not allow to complete the fitness tests thus the lack of available data gave an incomplete picture of the Viridiol effect on the olive fly. Further investigations would be necessary to explain this unusual result. No repulsive effect was detected, but the presence of proteins seemed to increase the toxic effect of the compound. The number of offspring in S-diet was statistically lower only in the most concentrated treatment (0.5%). The same result was obtained in (S+P)-diet. In summary, it was hypothesized that the higher concentration of Viridiol could act as symbioticides. This would confirm the antibiotic activity of this compound as already reported in literature (Sivasithamparam and Ghisalberti, 1998; Keswani *et al.*, 2014; Howell, 2003; Vinale *et al.*, 2006; 2014; Dias *et al.*, 2012; Chiang *et al.*, 2009; Mukherjee *et al.*, 2006; Pascale *et al.*, 2017). Anyhow, the

significantly higher rate of mortality in males fed on high-protein diet suggested the occurrence of a toxic effect of Viridiol.

- Harzianic Acid administration did not affect the mortality rate of females regardless of the diet type. For males fed on (S+P)-diet a significantly higher rate of mortality was observed in the most concentrated treatment. Thus, as well as for Viridiol, *B. oleae* males showed a lower tolerance threshold to this compound than females but in a concentration-dependent manner. No repulsive effect was detected, while in the particular case of Harzianic Acid 0.05% in (S+P)-diet a significantly higher quantity of diet was ingested by males, showing that in lower concentration the compound could act as attractive for males. A concentration-dependent effect on females' fecundity was detected even if the pattern was not easy to interpret since at different concentration opposite effects were observed. The decrease of the number of eggs laid by females could be explained by a possible toxic effect of the high-concentrated treatment when administered in addition with proteins. In S-diet the mortality rate, amount of ingested diet and the number of laid eggs did not differ from the control whereas in high-protein diet some of these parameters were statistically different. In (S+P)-diet, both the medium- and high-concentrated treatments showed a significantly lower amount of offspring. Thus, Harzianic Acid seemed to have a symbioticides effect on *B. oleae* adults that would lead to a higher larval mortality and this effect appears to be related to the treatment concentration and the diet type. The antibiotic activity of Harzianic Acid is well-known and these results seem to confirm it and to endorse that this compound may act symbioticides on "*Ca.* Erwinia dacicola" (Sivasithamparam and Ghisalberti, 1998; Keswani *et al.*, 2014; Howell, 2003; Vinale *et al.*, 2006; 2014; Dias *et al.*, 2012; Chiang *et al.*, 2009).

- 6-pentyl-α-pyron did not affect the mortality rate of both sexes when administered without proteins. On the contrary, in (S+P)-diet, the high-concentrated treatment led to a significantly higher mortality. Females apparently have a lower tolerance threshold to this compound depending on the treatment concentration and the diet type. The high-concentrated treatment act as a repellent for both sexes. The number of laid eggs was not affected by 6-pentyl-α-pyron treatment when it was administered in S-diet, while a lower amount of laid eggs was recorded in (S+P)-diet but only in the lower concentration. Therefore, the higher mortality rate of females and the lower amount of laid eggs

suggested a possible toxic effect of the compound depending on the presence of proteins in the diet. The number of offspring per female was significantly low in all the concentrations and in both the diet type. This result supports the hypothesis that the oral administering of 6-pentyl-α-pyron had a strong symbioticides effect on the endosymbiotic bacteria of the olive fruit fly. This would confirm the antibiotic activity of this compound as already reported in literature (Sivasithamparam and Ghisalberti, 1998; Keswani *et al.*, 2014; Howell, 2003; Vinale *et al.*, 2006; 2014; Dias *et al.*, 2012; Chiang *et al.*, 2009; Mukherjee *et al.*, 2006; Pascale *et al.*, 2017).

- Lipopeptides extracted from *B. subtilis* administration did not affect the mortality rate of both sexes when administered without proteins. On the contrary, in (S+P)-diet, the high-concentrated treatment led to a significantly higher mortality in both sexes. A reduced daily intake was observed for the most concentrated treatment, thus it might be hypothesized a repellent effect neutralized when the proteins are added to the diet. The lack of data available for the (S+P)-diet for fertility and offspring gave an incomplete picture of the role of the proteins on Lipopeptides effect on the olive fly fitness. The recorded decrease of offspring was directly proportional to the treatment concentration. The fact that the treatment did not affect the number of laid eggs and that the number of offspring was significantly lower, suggested a symbioticide effect of the compound in a concentration-dependent manner. This would confirm the antibiotic activity of this compound as already reported in literature (Asaka and Shoda 1996; Chen and Wu 1999; Harris and Adkins 1999; Ferreira *et al.* 1991; Sholberg *et al.* 1995; Mari *et al.* 1996; Raaijmakers *et al.* 2002; He *et al.* 1994).

5.2 ENDOSYMBIOTIC BACTERIA QUANTIFICATION BY REAL-TIME PCR

5.2.1 MICROBIOME ALTERATION IN MID-GUT

The bacterial quantification achieved by real-time PCR showed that most of the treatments did not affect the bacteria inhabiting the mid-gut of the treated females. A significant decrease was obtained only with Antibiotics in both the diet type experiments and in Viridiol 0.1% but only in

S-diet (Figure 26). On the contrary, a significant increase of the bacterial titer was obtained in Copper Oxychloride 0.02% treatment in (S+P)-diet (Figure 28).

Taking into account that the mid-gut of tephritids is known as inhabited by several species of bacteria, it was hypothesized that the symbioticide effect on "*Ca.* Erwinia dacicola" might be masked by the proliferation of opportunistic bacterial species. In fact, while the couple of primers utilized for real-time PCR could be considered as specific in the oesophageal bulb environment, it was not possible to rule out that in mid-gut samples also other bacterial species were amplified by primers, thus altering the results of the endosymbiotic bacteria quantification.

The oesophageal bulb is a characteristic structure with marked morphological differences that diverges from all the other species of the Tephritidae family. That can be explained by a coevolution with its specific symbiotic bacterial species (Capuzzo *et al.*, 2005; Mazzon *et al.*, 2008. 2010; Estes *et al.*, 2009). From literature "*Ca.* Erwinia dacicola" predominantly inhabits this organ and it is unlikely that in case of decrease of the specific endosymbiont other bacteria can colonize it. On the contrary, in the mid-gut numerous species of opportunistic bacteria might proliferate if the presence of the specific endosymbiont decrease as already reported in several studies (Estes *et al.*, 2012; Ben-Yosef *et al.*, 2015. Ras *et al.*, 2017).

Further metagenomics analysis on mid-gut samples could clarify the composition of bacterial microbiome corresponding to each treatment and verify if a replacement of the beneficial bacteria for opportunistic species may occur. This hypothesis could explain why the bacterial quantification did not detect a significant decrease of the endosymbiont titer even in those treatments where a significant fitness reduction of the olive fly was observed.

5.2.2 MICROBIOME ALTERATION IN OESOPHAGEAL BULB

The bacterial quantification achieved by real-time PCR showed that most of the treatments led to a significant reduction of the bacteria inhabiting the oesophageal bulb of the treated females (Figure 30 and Figure 32).

The presence of protein in the diet intensifies the symbioticide effect of the administered compounds. It was particularly clear in Antibiotics treatment where the bacterial titer reduction was more than 2 times higher in comparison with the S-diet counterparts. The same pattern was observed for all other treatments were both the diet type data were available (Figure 33). The

molecular analysis too showed that the nutritional condition of the fly might play a role in the bacterial reduction.

The molecular analysis on oesophageal bulb confirmed the symbioticide effect of Viridiol, Harzianic Acid and Copper Oxychloride in the lower concentrated treatment. Unfortunately, it was not possible to verify the bacterial titer of Lipopeptides and 6-pentyl-α-pyron treatments, since a storage problem of the DNA samples occurred.

6 CONCLUSIONS

In conclusion, the results obtained confirmed that "*Ca.* Erwinia dacicola" plays an essential role in *B. oleae* larval development in unripe olive fruits (Ben-Yosef *et al.*, 2015). In adult fitness, the role of the bacterium is marginal when the diet administered totally lack of nitrogen sources (S-diet) or is fully provided of all the essential amino acids (S+P diet) (Ben-Yosef *et al.*, 2010; 2014).

Furthermore, the present study demonstrated that the oral administration of some microbial metabolites such as Viridiol and Harzianic Acid could act as symbioticides and affect the *B. oleae* fitness. The antibiotic activity of these microbial metabolites is well known and widely reported in the scientific literature. In fact, their application is addressed against plant pathogens as Microbial Biological Control Agents due to antifungal and amending properties (Sivasithamparam and Ghisalberti, 1998; Keswani *et al.*, 2014; Howell, 2003; Vinale *et al.*, 2006; 2014; Dias *et al.*, 2012; Chiang *et al.*, 2009; Mukherjee *et al.*, 2006; Pascale *et al.*, 2017). To our knowledge, this is the first study in which the efficacy of the antibacterial activity of these compounds has been evaluated against primary endosymbiotic bacteria of an insect species.

The same effect was in part obtained also with the oral administration of Copper Oxychloride, which has shown a strong toxic and a moderate symbioticide effect that, anyhow, affect both the adult and larval fitness of the olive fly. This would confirm the previous study about the toxicity of Copper for insects in a concentration-dependent manner (Jensen and Trumble, 2003; Cheruiyot 2012; Cheruiyot *et al.*, 2013), but also its possible symbioticide effect on olive fly endosymbiont (Rosi *et al.*, 2007; Sacchetti *et al.*, 2004; Belcari and Bobbio, 1999; Tzanakakis, 1985).

Lipopeptides extracted from *B. subtilis* and 6-pentyl-α-pyron resulted to be promising compounds that could act as symbioticides but further molecular analyses are required to confirm their actual effectiveness on "*Ca.* Erwinia dacicola".

The antibiotic properties of Lipopeptides extracted from *B. subtilis* have been widely reported in literature and successfully used for the biocontrol of several plant diseases (Asaka and Shoda 1996; Chen and Wu 1999; Harris and Adkins 1999; Ferreira *et al.* 1991; Sholberg *et al.* 1995; Mari *et al.* 1996; Raaijmakers *et al.* 2002; He *et al.* 1994). To our knowledge, this is the first

study in which the efficacy of the antibacterial activity of Lipopeptides from *B. subtilis* has been evaluated against primary endosymbiotic bacteria of an insect pest.

The antimicrobial activity of 6-pentyl-α-pyron is well known and widely reported in literature but, its application was limited as Microbial Biological Control Agents against plant pathogens (Sivasithamparam and Ghisalberti, 1998; Keswani *et al.*, 2014; Howell, 2003; Vinale *et al.*, 2006; 2014; Dias *et al.*, 2012; Chiang *et al.*, 2009; Mukherjee *et al.*, 2006; Pascale *et al.*, 2017). An acaricidal activity was also reported by Salwa Sholla and Metwally Kottb (2017) against the two-spotted spider mite *Tetranychus urticae*. To our knowledge, this is the first study in which the efficacy of the antibacterial activity of 6-pentyl-α-pyron has been evaluated against primary endosymbiotic bacteria of an insect species.

Evidence provided by this study seem to be consistent with previous studies in which a tolerance decrease to microbial metabolites was observed in combination with high-protein diet (Shikano and Cory, 2014; Abdullah *et al.*, 2017).

The present study represents the first step for the identification of environmentally friendly compounds that may be used as symbiosis inhibitors that could lead to the development of new formulations for the olive fruit fly integrated pest management.

7 REFERENCES

Abdullah E, Idris A, Saparon A. (2017). Resistance and tolerance: the role of nutrients on pathogen dynamics and infection outcomes in an insect host. *ARPN J. Eng. Appl. Sci. 12*(10): 3218–3221. (https://doi.org/10.1111/ijlh.12426)

Alagna F, Mariotti R, Panara F, Caporali S, Urbani S, Veneziani G, Esposto S, Taticchi A, Rosati A, Rao R, Perrotta G, Servili M, Baldoni L. (2012). Olive phenolic compounds: metabolic and transcriptional profiling during fruit development. *BMC Plant Biol.* 12: 162. (doi:10.1186/1471-2229-12-162)

Alzaghal K, Mustafa T. (1987). Studies on the pupation of the olive fruit fly, *Dacus oleae* Gmel (Diptera, Tephritidae), in Jordan. *J. Appl. Entomol*. 103: 452–56

Amiot MJ, Fleuriet A, Macheix JJ. (1986). Importance and evolution of phenolic compounds in olive during growth and maturation. *J. Agric. Food Chem*. 34. 823–826. (doi:10.1021/jf00071a014)

Angerosa F, Di Giacinto L, Solinas M. (1992). Influence of *Dacus oleae* infestation on flavor of oils, extracted from attacked olive fruits, by HPLC and HRGC analyses of volatile compounds. *Grasas y Aceites* 43: 134-142. [91]

Angiolillo A, Mencuccini M, Baldoni L. (1999). Olive genetic diversity assessed using amplified fragment length polymorphisms. *Theor. Appl. Genet.* 98: 411–421.

Apostolaki A, Livadaras I, Saridaki A, Chrysargyris A, Savakis C, Bourtzis K. (2011). Transinfection of the olive fruit fly *Bactrocera oleae* with *Wolbachia*: Towards a symbiont-based population control strategy. *J. Appl. Entomol.* 135: 546–553. (http://doi.org/10.1111/j.1439-0418.2011.01614.x).

Arthofer W, Riegler M, Schneider D, Krammer M, Miller WJ, Stauffer C. (2009). Hidden *Wolbachia* diversity in field populations of the European cherry fruit fly, *Rhagoletis cerasi* (Diptera, Tephritidae). *Mol. Ecol.* 18. 3816–3830.

Asaka O, Shoda M. (1996). Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. *Appl. Environ. Microbiol.* 62: 4081–4085.

Baker R, Herbert R, Howse PE, Jones OT, Francke W, Reith W. (1980). Identification and synthesis of the major sex pheromone of the olive fruit fly (*Dacus oleae*). *J. Chem. Soc. Chem. Commun*. 1:52–53

Baldoni L, Guerrero C, Sossey-Aloui K, Abbott AG, Angiolillo A, Lumaret R. (2002). Phylogenetic relationships among *Olea* species, based on nucleotide variation at a non-coding chloroplast DNA region. *Plant Biol*. 4. 346–351.

Barras F, van Gijsegem F, Chatterjee AK. (1994). Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. *Annu. Rev. Phytopathol*. 32: 201–234. (doi:10.1146/annurev.py.32.090194.001221)

Bateman MA. (1972). The ecology of fruit flies. *Annu. Rev. Entomol*. 17: 493–518.

Baumann P, Moran NA. (1997). Non-cultivable microorganisms from symbiotic associations of insects and other hosts. *Antonie van Leeuwenhoek* 72. 39–48.

Baumann P. (2005). Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu. Rev. Microbiol.* 59: 155-189.

Behar A, Ben-Yosef M, Lauzon CR, Yuval B, Jurkevich E. (2009). Structure and function of the bacterial community associated with the Mediterranean fruit fly. In Insect symbiosis (eds K Bourtzis, TA Miller), pp.251–271. *Boca Raton, FL: CRC Press*.

Behar A, Jurkevitch E, Yuval B. (2008). Bringing back the fruit into fruit fly–bacteria interactions. *Mol. Ecol.* 17. 1375–1386. (doi:10.1111/j.1365-294X. 2008.03674.x)

Behar A, Yuval B, Jurkevitch E. (2005). Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly *Ceratitis capitata*. *Mol. Ecol.* 14. 2637– 2643.

Belcari A, Bobbio E. (1999). L'impiego del rame nel controllo della mosca delle olive, *Bactrocera oleae*. *Inf. Fitopatol*. 12. 52–55.

Belcari A, Sacchetti P, Marchi G, Surico G. (2003). La mosca delle olive e la simbiosi batteri*Ca. Inf. Fitopatol*. 9. 55–59.

Belcari A, Sacchetti P, Rosi MC, Del Pianta R. (2005). Control of the olive fly (*Bactrocera oleae*) through the use of copper products in Central Italy. *IOBC/WPRS Bull*. 28. 45–48.

Belcari A. (1989). Contributi alla conoscenza dei Ditteri Tefritidi. IV. *Frust. Ent*. 10: 83-125.

Benitez T, Rincon AM, Limon MC, Codon AC. (2004). Biocontrol mechanisms of *Trichoderma* strains. *Int Microbiol*. 7(4): 249- 60.

Bennett RN, Wallsgrove RM. (1994). Secondary metabolites in plant defence mechanisms. *New Phytol.* 127. 617–633. (doi:10.1111/j.1469-8137.1994. tb02968.x)

Ben-Yosef M, Aharon Y, Jurkevitch E, Yuval B. (2010). Give us the tools and we will do the job: symbiotic bacteria affect olive fly fitness in a diet-dependent fashion. *P. Roy. Soc. B: Bio. Sci.* 277: 1545–1552.

Ben-Yosef M, Pasternak Z, Jurkevitch E, Yuval B. (2015). Symbiotic bacteria enable olive fly larvae to overcome host defenses. *Roy. Soc. Open Sci.* 2(7): 150170. http://doi.org/10.1098/rsos.150170

Ben-Yosef M, Pasternak Z, Jurkevitch E, Yuval B. (2014). Symbiotic bacteria enable olive flies (*Bactrocera oleae*) to exploit intractable sources of nitrogen. *J. Evol. Biol*. 27: 2695–2705. doi: 10.1111/jeb.12527

Besnard G, Rubio de Casas R, Christin PA, Vargas P. (2009). Phylogenetics of *Olea* (Oleaceae) based on plastid and nuclear ribosomal DNA sequences: tertiary climatic shifts and lineage differentiation times. *Ann. Bot*. 104: 143–160.

Besnard G, Rubio de Casas R, Vargas P, (2007). Plastid and nuclear DNA polymorphism reveal historical processes of isolation and reticulation in the olive tree complex (*Olea europea*). *J. Biogeogr*. 34: 736–752.

Boardman J. (1976). The olive in the Mediterranean: its culture and use. *Philos. Trans. R. Soc. Lond., B, Biol. Sci*. 275: 187–196.

Boush MG, Matsumura F. (1967). Insecticidal degradation by *Pseudomonas melophthora*, the bacterial symbiote of the apple maggot. *J. Econ. Entomol*. 60: 918–920 (913). doi:10.1093/jee/60. 4.918

Braendle C, Miura T, Bickel R, Shingleton AW, Kambhampati S, Stern DL. (2003). Developmental origin and evolution of bacteriocytes in the aphid-*Buchnera* symbiosis. *PLoS Biol*. 1: E21.

Brenes M, Garcia A, De los Santos B, Medina E, Romero CN, De Castro A, Romero F. (2011). Olive glutaraldehyde-like compounds against plant pathogenic bacteria and fungi. *Food Chem.* 125: 1262–1266. (doi:10.1016/j.foodchem.2010. 10.055)

Briante R, Patumi M, Limongelli S, Febbraio F, Vaccaro C, Di Salle A, La Cara F, Nucci R. (2002) Changes in phenolic and enzymatic activities content during fruit ripening in two Italian cultivars of *Olea europaea* L. *Plant Sci.* 162: 791–798. (doi:10.1016/S0168-9452(02)00022-5)

Broumas T. (1985). Application trials of trapping systems for control of *Dacus oleae*. *Ann. Inst. Phytopathol*. Benaki (N.S.) 14:157–66

Brune A, Dietrich C. (2015). The gut microbiota of termites: Digesting the diversity in the light of ecology and evolution. *Annu. Rev. Microbiol.* 69: 145-166.

Buchner P. (1965). Endosymbiosis of animals with plant microorganisms. *Interscience Publishers*, New York.

Burrack HJ, Zalom FG. (2008). Olive fruit fly (Diptera: Tephritidae) ovipositional preference and larval performance in several commercially important olive varieties in California. *J. Econ. Entomol.* 101: 750–58

Caleca V, Lo Verde G, Lo Verde V, Palumbo Piccionello M, Rizzo R. (2010). Control of *Bactrocera olea*e and *Ceratitis capitata* in Organic Orchards: Use of Clays and Copper Products. Proc. Organic Fruit Conference (Prange e Bishop S. D. coord.). *Acta Horticulturae,* 873: 227-233.

Canale A, Carpita A, Conti B, Canovai R, Raspi A. (2012). Effect of age on 1.7-dioxaspiro-[5.5]-undecane production in both sexes of olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae). *IOBC Bull.* 72: 219–225.

Capuzzo C, Firrao G, Mazzon L, Squartini A, Girolami V. (2005). "*Candidatus* Erwinia dacicola", a co-evolved symbiotic bacterium of the olive fly *Bactrocera oleae* (Gmelin). *Int. J. Syst. Evol. Microbiol*. 55: 1641-1647

Cejanavarro JA, Vega FE, Karaoz U, Zhao H, Jenkins S, Lim HC, Kosina P, Infante F, Northen TR, Brodie EL. (2015). Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. *Nat Commun*. 6:7618.

Chen TW, Wu WS. (1999). Biological control of carrot black rot. *J. Phytopathol.* 147: 99–104.

Cheng D, Guo Riegler M, Xi Z, Liang G, Xu Y. (2017). Gut symbiont enhances insecticide resistance in a significant pest, the oriental fruit fly *Bactrocera dorsalis* (Hendel). *Microbiome* 5:13. DOI http://doi.org/10.1186/s40168-017-0236-z

Cheng Q, Aksoy S. (1999). Tissue tropism, transmission, and expression of foreign genes in vivo in midgut symbionts of tsetse flies. *Insect Mol. Biol*. 8:125–132.

Cheruiyot DJ, Boyd RS, Coudron TA, Cobine PA. (2013). Biotransfer, Bioaccumulation, and Effects of Herbivore Dietary Co, Cu, Ni, and Zn on Growth and Development of the Insect Predator *Podisus maculiventris* (Say). *Journal of Chemical Ecology*, *39*(6): 764–772. https://doi.org/10.1007/s10886-013-0289-9

Cheruiyot DJ. (2012). Investigation of elemental defense and trophic transfer of metals using beet armyworm, *Spodoptera exigua*, Hübner. Ph.D. dissertation. *Auburn University*, *Alabama*

Chiang Y, Lee K, Sanchez JF, Keller NP, Wang CC. (2009). Unlocking fungal cryptic natural products. *Nat. Prod. Commun*. 4: 1505–1510.

Cochran, D. (1985). Nitrogenous excretion. In: Comprehensive Insect Physiology, Biochemistry, and Pharmacology, Vol. 4 (G.A. Kerkurt and L.I. Gilbert, eds), pp. 467–506. *Pergamon Press*, Oxford.

Corrado G, Garonna A, Cabanas CG-L, Gregoriou M, Martelli GP, Mathiopoulos KD. (2016). Host Response to Biotic Stresses. In: Rugini E, Baldoni L, Muleo R, Sebastiani L, editors. The live tree Genome. *Springer International Publishing*; p. 75-98.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. (1995). Microbial biofilms. *Annu. Rev. Microbiol*. 49: 711-745.

Currie CR, Wong B, Stuart AE, Schultz TR, Rehner SA, Mueller UG, Sung GH, Spatafora JW, Straus NA. (2003). Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science.* 299: 386–388.

Daane KM, Johnson MW. (2010). Olive fruit fly: managing an ancient pest in modern times. *Annu. Rev. Entomol.* 55: 151–169. (https://doi.org/10.1146/annurev.ento.54.110807.090553).

Daane KM, Rice RE, Zalom FG, Barnett WW, Johnson MW. (2005). Arthropod pests of olive. In Olive Production Manual, ed. GS Sibbit, L Ferguson, pp. 105–14. *Oakland, CA: Univ. California Agric. Nat. Res.*

Dadd RH. (1985). Nutrition: organisms. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 4 (G.A. Ker- kurt and L.I. Gilbert, eds), pp. 313–390. *Pergamon Press, Oxford.*

Dale C, Moran NA. (2006). Molecular interactions between bacterial symbionts and their hosts. *Cell*. 126: 453–465.

Davey ME, O'Toole GA. (2000). Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev*. 64:847–867.

Davis RE. (1927). The nitrogenous constituents of hen urine. *J. Biol. Chem*. 74: 509–513.

Dedeine F, Bandi C, Boule´treau M, Kramer L. (2003). Insights into *Wolbachia* obligatory symbiosis. In: Insect symbiosis. Ed. by Bourtzis K, Miller T, *CRC Press, Boca Raton, Florida, USA*, 267–282.

Delrio G, Cavalloro R. (1977). Observations on the life cycle and the population dynamics *of Dacus oleae* Gmel. in Liguria. *Redia* 60:221–53

Delucchi V. (1957). Les parasites de la mouche des olives. *Entomophaga* 2:107–18

Dias DA, Urban S, Roessner U. (2012). A Historical Overview of Natural Products in Drug Discovery. *Metabolites*. 2: 303–336.

Dìaz-Fleischer F, Aluja M. (2001). Behavior of tephritid flies: a historic perspective. In Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior, ed. MAluja, AL Norrbom, pp. 36–69. Boca Raton, FL: *CRC Press*

Diaz-Fleischer F, Papaj DR, Prokopy RJ, Norrbom AL, AlujaM, Aluja M, Norrbom AL. (2000). Evolution of fruit fly oviposition behavior. In Fruit flies (Tephritidae): phylogeny and evolution of behavior (ed. MAluja, AL Norrbom), pp. 811–842. Boca Raton, FL: *CRC Press.*

Dillon RJ, Dillon VM. (2004). The gut bacteria of insects: nonpathogenic interactions. *Annu. Rev. Entomol.* 49:71–92.

Dobler S, Petschenka G, Pankoke H. (2011). Coping with toxic plant compounds—the insect's perspective on iridoid glycosides and cardenolides. *Phytochemistry* 72: 1593–1604. (doi:10.1016/j.phytochem.2011.04.015)

Donia AR, El-Sawaf SK, Abou-Ghadir MF. (1971). Number of generations and seasonal abundance of the olive fruit fly, *Dacus oleae* Gmelin, and the susceptibility of different olive varieties to infestation. *Bull. Soc. Entomol*. Egypt 55:201–9

Douglas A.E. (1998). Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria *Buchnera*. *Annu. Rev. Entomol.* 43: 17–37.

Douglas AE, Minto LB, Wilkinson TL. (2001). Quantifying nutrient production by the microbial symbionts in an aphid. *J. Exp. Biol.* 204: 349- 358.

Douglas AE. (2006). Phloem-sap feeding by animals: problems and solutions. *J. Exp. Bot.* 57: 747–754.

Douglas AE. (2009). The microbial dimension in insect nutritional ecology. *Funct. Ecol.* 23:38-47.

Douglas AE. (2013). Microbial brokers of insect-plant interactions revisited. *J. Chem. Ecol.* 39: 952-961.

Dowd PF. (1992) Insect fungal symbionts: a promising source of detoxifying enzymes. *J. Ind. Microbiol.* 9: 149–161. (doi:10.1007/BF01569619)

Drew RAI, and B. Yuval. (2000). The evolution of fruit fly feeding behavior, p. 731–749. In M. Aluja and A. L. Norrbom (ed.), Fruit flies (Tephritidae): phylogeny and evolution of behavior. *CRC Press,* Boca Raton, FL.

Drew RAI, Courtice AC, Teakle DS. (1983). Bacteria as a natural source of food for adult fruit flies (Diptera: Tephritidae). *Oecologia* (Berl.) 60: 279-284.

Drew RAI, Faye HA. (1988). Elucidation of the roles of ammonia and bacteria in the attraction of *Dacus tryoni* (Froggatt) (Queensland fruit fly) to proteinaceous suspensions. *J. Plant. Prot. Trop.* 5: 127-130.

Drew RAI, Lloyd AC. (1987). Relationship of fruit flies (Diptera: Tephritidae) and their bacteria to host plants. *Annu. Entomol. Soc. Am.* 80: 629-636.

Drew RAI, Lloyd AC. (1991). Bacteria in the life cycle of Tephritid fruit flies, p. 441–465. In P. Barbosa, V. A. Krischik, and C. G. Jones (ed.), Microbial mediation of plant-herbivore interactions. *John Wiley and Sons, Inc*., New York, NY.

Dunbar HE, Wilson AC, Ferguson NR, Moran NA. (2007). Aphid thermal tolerance is governed by a point mutation in bacterial symbionts. *PLoS Biol.* 5: 1006–15 (doi:10.1371/journal.pbio.0050096)

Economopoulos AP, Zervas GA. (1982). The quality problem in olive flies produced for SIT experiments: *IAEA STI/PUB.*

Economopoulos AP. (1972). Sexual competitiveness of gamma-ray sterilized males of *Dacus oleae*. Mating frequency of artificially reared and wild females. *Environ. Entomol.* 1: 490–497.

Economopoulos AP. (2002). The olive fruit fly, *Bactrocera* (*Dacus*) *oleae* (Gmelin) (Diptera: Tephritidae): its importance and control; previous SIT research and pilot testing. *IAEA STI/PUB.*

Engel P, Moran NA. (2013). The gut microbiota of insects diversity in structure and function. *FEMS Microbiol. Rev.* 37: 699–735.

Engelstädter J, Hurst GDD. (2009). The ecology and evolution of microbes that manipulate host reproduction. *Annu. Rev. Ecol. Evol. Syst.* 40: 127-149.

Epsky ND, Heath RR, Dueben BD, Lauzon CR, Proveaux AT, MacCollom GB. (1998). Attraction of 3-methyl-1-butanol and ammonia identified from Enterobacter agglomerans to *Anastrepha suspensa*. *J. Chem. Ecol.* 24: 1867-1880.

Estes AM, Hearn DJ, Burrack HJ, Rempoulakis P, Pierson EA. (2012a). Prevalence of *Candidatus* Erwinia dacicola in wild and laboratory olive fruit fly populations and across developmental stages. *Environ. Entomol.* 41(2): 265–274. (http://doi.org/10.1603/EN11245).

Estes AM, Nestel D, Belcari A, Jessup A, Rempoulakis P, Economopoulos AP. (2012b). A basis for the renewal of sterile insect technique for the olive fly, *Bactrocera oleae* (Rossi). *J. Appl. Entomol.* 136: 1–16. (http://doi.org/10.1111/j.1439-0418.2011.01620.x).

Estes AM. (2009). Life in a fly: the ecology and evolution of the olive fly endosymbiont, *Candidatus* Erwinia dacicola. Ph.D. thesis, University of Arizona, Tucson, AZ, USA.

Felton GW, Gatehouse JA. (1996). Antinutritive plant defence mechanisms. In Biology of the insect midgut (eds MJ Lehane, PF Billingsley), pp. 373–416. London, UK: Chapman and Hall.

Ferreira JHS, Matthee FN, Thomas AC. (1991). Biological control of *Eutypa lata* on grapevine by an antagonistic strain of *Bacillus subtilis*. *Phytopathology* 81: 283–287.

Fletcher BS, Kapatos ET. (1983). The influence of temperature, diet and olive fruits on the maturation rates of female olive flies at different times of the year. *Entomol. Exp. Appl.* 33: 244–52

Fletcher BS, Pappas S, Kapatos E. (1978). Changes in ovaries of olive flies (*Dacus oleae* (Gmelin)) during summer, and their relationship to temperature, humidity and fruit availability. *Ecol. Entomol.* 3: 99–107

Fletcher BS. (1987). The biology of dacine fruit flies. *Annu. Rev. Entomol.* 32: 115–144. (doi:10.1146/annurev.en.32.010187.000555)

Fortenbaugh WW, Gutas D. (1992). Theophrastus: His Psychological, Doxographical, and Scientific Writings (Rutgers University Studies in Classical Humanities, V). New Brunswik, London, Transaction Publishers.

Franz G, Kerremans P, Rendon P, Hendrichs J. (1996). Development and application of genetic sexing systems for Mediterranean fruit fly based on temperature sensitive lethal, pp. 185-191. In B. A. McPheron and G. J. Steck [eds.], Fruit fly pests, a world assessment of their biology and management. *St. Lucie Press*, Delray Beach, FL.

Fry AJ, Palmer MR, Rand DM. (2004). Variable fitness effects of *Wolbachia* infection in *Drosophila melanogaster*. *Heredity* 93: 379–389.

Genta FA, Dillon RJ, Terra WR, Ferreira C. (2006). Potential role for gut microbiota in cell wall digestion and glucoside detoxification in *Tenebrio molitor* larvae. *J. Insect. Physiol.* 52(6): 593-601.

Gil R, Latorre A, Moya A. (2004). Bacterial endosymbionts of insects: insights from comparative genomics. *Environ. Microbiol.* 6: 1109-1122.

Girolami V. (1973). Reperti morfo-istologici sulle batteriosimbiosi del *Dacus oleae* Gmelin e di altri ditteri tripetidi, in natura e negli allevamenti su substrati artificiali. *Redia* 54: 269–294.

Girolami, V. (1983). Fruit fly symbiosis and adult survival: general aspects. In Fruit flies of economic importance (R. Cavalloro, Ed.), 74–76. Proceedings of the CEC/IOBC International Symposium, Athens, November 1982.

Giron D, Dedeine F, Dubreuil G, Huguet E, Mouton L, Outreman Y, Simon J. (2016a). Influence of microbial symbionts on plant-insect interactions. Advances in Botanical Research (Vol. 81). Elsevier Ltd. (http://doi.org/10.1016/bs.abr.2016.09.007).

Giron D, Glevarec G. (2014). Cytokinin-induced phenotypes in plant-insect interactions: Learning from the bacterial world. *J. Chem. Ecol.* 40: 826-835.

Giron D, Huguet E, Stone GN, Body M. (2016b). Insect-induced effects on plants and possible effectors used by galling and leaf-mining insects to manipulate their host plant. *J. Insect Physiol.* 84: 70-89.

Gjersing EL, Codd SL, Seymour JD, Stewart PS. (2005). Magnetic resonance microscopy analysis of advective transport in a biofilm reactor. *Biotech. Bioengineer.* 89: 822-834.

Grasso F, Coppola M, Carbone F, Baldoni L, Alagna F, Perrotta G, Pérez-Pulido AJ, Garonna A, Facella P, Daddiego L, Lopez L, Vitiello A, Rao R. (2017). The transcriptional response to the olive fruit fly (*Bactrocera oleae*) reveals extended differences between tolerant and susceptible olive (*Olea europaea* L.) varieties. *Vontas J, ed. PLoS ONE*. 12(8): e0183050. doi:10.1371/journal.pone.0183050.

Gomez-Caravaca AM, Cerretani L, Bendini A, Segura-Carretero A, Fernandez-Gutierrez A. (2008). Effects of fly attack (*Bactrocera oleae*) on the phenolic profile and selected chemical parameters of olive oil. *J. Agric. Food Chem*. 56: 4577–83.

Greany PD. (1989). Host plant resistance to tephritids: an under-exploited control strategy. In Fruit flies: their biology, natural enemies and control (eds AS Robinson, G Hooper), pp. 353–362. Amsterdam, The Netherlands: Elsevier.

Greenberg B. (1959). Persistence of bacteria in the developmental stages of the housefly. III. Quantitative distribution in prepupae and pupae. *Am. J. Trop. Med. Hyg*. 8: 613–617.

Gucci R, Caruso G, Canale A, Loni A, Raspi A, Urbani S, (2012). Qualitative changes of olive oils obtained from fruits damaged by *Bactrocera oleae* (Rossi). *HortScience*. 47(2): 301-6.

Gumusay B, Ozilbey U, Ertem G, Oktar A. (1990). Studies on the susceptibility of some important table and oil olive cultivars of Aegean region to olive fruit fly (*Dacus oleae* Gmel.) in Turkey. *Acta Hortic.* 286: 359–61

Gutierrez-Rosales F, Romero MP, Casanovas M, Motilva MJ, Minguez-Mosquera MI. (2012). β-Glucosidase involvement in the formation and transformation of oleuropein during the

growth and development of olive fruits (*Olea europaea* L. cv. Arbequina) grown under different farming practices. *J. Agric. Food Chem.* 60: 4348–4358. (doi:10.1021/ jf205209y)

Hanhong B. (2011). *Trichoderma* species as abiotic and biotic stress quenchers in plants. *Res J Biotechnol*; 6(3): 73-9.

Haniotakis GE, Kozyrakis E, Bonatsos C. (1986). Control of the olive fruit fly, *Dacus oleae* Gmel (Dipt., Tephritidae), by mass trapping—pilot scale feasibility study. *J. Appl. Entomol.* 101: 343–52

Haniotakis GE, Mazomenos BE, Tumlinson JH. (1977). Sex attractant of olive fruit fly, *Dacus oleae,* and its biological activity under laboratory and field conditions. *Entomol. Exp. Appl.* 21: 81–87

Harris AR, Adkin PG. (1999). Versatility of fungal and bacterial isolates for biological control of damping-off disease caused by *Rhizoctonia solani* and *Pythium* spp. *Biol. Control* 15: 10–18.

He H, Silo-Suh LA, Handelsman J. (1994). Zwittermicin A, an antifungal and plant protection agent from *Bacillus cereus*. *Tetrahedron letters* 35: 2499–2502.

Headrick DH, Goeden RD. (1998). The biology of nonfrugivorous tephritid fruit flies. *Annu. Rev. Entomol.* 43: 217–241.

Hellmuth H. (1956). Untersuchungen zur bakteriensymbiose der trypetiden (Diptera). *Zoomorphology* 44: 483–517.

Hendrichs J, Lauzon CR, Cooley SS, Prokopy RJ. (1993). Contributions of natural food sources to adult longevity and fecundity in *Rhagoletis pomonella* (Diptera: Tephritidae). *Ann. Entomol. Soc. Am.* 86: 250-264.

Hoffmann AA, Turelli M. (1997). CI in insects. In: Influential passengers: inherited microorganisms and arthropod reproduction. Ed. by O'Neill SL, Hoffmann AA, Werren JH, *Oxford University Press*, Oxford, UK, 42–80.

Howell CR. (2003). Mechanisms employed by *Trichoderma species* in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis.* 87: 4-10.

Hurst GDD, Jiggins FM, von der Schulenburg JHG, Bertrand D, West SA, Coriachera II, Zakharov IA, Werren JH, Stouthamer R, Majerus MEN. (1999). Male-killing *Wolbachia* in two species of insects. *Proc. R. Soc. Lond. B Biol. Sci*. 266: 735–740.

Iannotta N, Noce ME, Ripa V, Scalercio S, Vizzarri V. (2007). Assessment of susceptibility of olive cultivars to the *Bactrocera oleae* (Gmelin, 1790) and *Camarosporium dalmaticum* (Thum.) attacks in Calabria (Southern Italy). *J. Environ. Sci. Health Part B* 42: 789–93

Iannotta N, Scalercio S. (2012). Susceptibility of Cultivars to Biotic Stresses. In: Mazzalupo I, editor. Olive Germplasm. The Olive Cultivation, Table Olive and Olive Oil Industry in Italy: INTECH Open Access Publisher: p. 81-106.

Iannotta, N. (2004). La difesa dell'olivo dai principali fitofagi con metodi conformi al Reg. CEE 2092/91. – In: La difesa dai fitofagi in condizioni di olivicoltura biologi*Ca*. Atti Accademia nazionale dell'Olivo e dell'Olio, Spoleto, 29-30 October 2002: 49-62.

Ishikawa H. (2003). Insect symbiosis: an introduction. p. 1–16. In K. Bourtzis and T. Miller (ed.), Insect symbiosis. *CRC Press*, Boca Raton, FL.

Janson EM, Stireman JO, Singer MS, Abbot P. (2008). Phytophagous insect-microbe mutualisms and adaptive evolutionary diversification. *Evolution* 62: 997-1012.

Jensen P, Trumble JT. (2003). Ecological consequences of bioavailability of metals and metalloids. *Recent Res. Dev. Entomol*. 4:1–17

Jeyaprakash A, Hoy MA. (2000). Long PCR improves DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol*. 9: 393–405.

Jiang C, Baehrecke EH, Thummel CS. (1997). Steroid regulated programmed cell death during *Drosophila* metamorphosis. *Development* 124: 4673–4683.

Joachim-Bravo IS, Fernandes OA, Bortoli SRA, Zucoloto FS. (2001). Oviposition behavior of *Ceratitis capitata* Wiedemann (Diptera: Tephritidae): association between oviposition preference and larval performance in individual females. *Neotrop. Entomol*. 30: 559–564. (doi:10.1590/S1519- 566X2001000400008)

Joubert DA, O'Neill SL. (2017). Comparison of stable and transient *Wolbachia* infection models in *Aedes aegypti* to block dengue and west Nile, (January). PLoS Neglected Tropical Diseases 4: 1-14. http://doi.org/10.1371/journal.pntd.0005275

Kaiser W, Huguet E, Casas J, Commin C, Giron D. (2010). Plant green-island phenotype induced by leaf-miners is mediated by bacterial symbionts. *P. Roy. Soc. Lond. B. Bio.* 277: 2311-2319.

Kakani EG, Mathiopoulos KD. (2008). Organophosphate resistance-related mutations in the acetyl- cholinesterase gene of Tephritidae. *J. Appl. Entomol.* 132: 762–71

Kaltenpoth, M. (2009). Actinobacteria as mutualists: General healthcare for insects? *Trends Microbiol.* 17: 529–535.

Kapatos E, Fletcher BS, Pappas S, Laudeho Y. (1977). Release of *Opius concolor* and *Opius concolor* var siculus (Hym.: Braconidae) against spring generation of *Dacus oleae* (Dipt.: Trypetidae) on Corfu. *Entomophaga* 22: 265–70

Kapatos ET, Fletcher BS. (1984). The phenology of the olive fly, *Dacus oleae* (Gmel.) (Diptera, Tephritidae), in Corfu. *Z. Angew. Entomol.* 97: 360–370. (doi:10.1111/j.14390418.1984.tb03760.x)

Katsoyannos BI, Kouloussis NA. (2001). Captures of the olive fruit fly *Bactrocera olea*e on spheres of different colors. *Entomol. Exp. Appl.* 100:165–72

Katsoyannos BI. (1989). Response to shape, size, and color. InWorld Crop Pests: Fruit Flies—Their Biology, Natural Enemies and Control, ed. AS Robinson, G Hooper, 3A:307–24. Amsterdam: Elsevier

Katsoyannos P. (1992). Olive pest and their control in the Near East. FAO Plant production and protection paper 115. FAO Rome, Rome

Kaufman MG, Walker ED, Odelson DA, Klug MJ. (2000). Microbial community ecology and insect nutrition. *Am. Entomol*. 46: 173–185.

Keswani C, Mishra S, Sarma BK, Singh SP, Singh HB. (2014). Unraveling the efficient applications of secondary metabolites of various *Trichoderma* spp. *Appl. Microbiol. Biotechnol.* 98: 533–544.

Kikuchi Y, Hayatsu M, Hosokawa T, Nagayama A, Tago K, Fukatsu T. (2012). Symbiont-mediated insecticide resistance. *Proc. Natl. Acad. Sci. USA*. 109(22): 8618–22.

Kikuchi Y, Hosokawa T, Nikoh N, Meng XY, Kamagata Y, Fukatsu T. (2009). Host-symbiont co-speciation and reductive genome evolution in gut symbiotic bacteria of acanthosomatid stinkbugs. *BMC Biology* 7.

Kittayapong P, Milne JR, Tigvattananont S, Baimai V. (2000). Distribution of the reproduction-modifying bacteria, *Wolbachia*, in natural populations of tephritid fruit flies in Thailand. *ScienceAsia* 26: 93- 103.

Klepzig KD, Adams AS, Handelsman J, Raffa KF. (2009). Symbioses: A key driver of insect physiological processes, ecological interactions, evolutionary diversification, and impacts on humans. *Environ Entomol* 38:67-77.

Konno K, Hirayama C, Shinbo H, Nakamura M. (2009). Glycine addition improves feeding performance of non-specialist herbivores on the privet, *Ligustrum obtusifolium*: in vivo evidence for the physiological impacts of anti-nutritive plant defense with iridoid and insect adaptation with glycine. *Appl. Entomol. Zool.* 44: 595–601. (doi:10.1303/aez.2009.595)

Konno K, Hirayama C, Yasui H, Nakamura M. (1999). Enzymatic activation of oleuropein: a protein crosslinker used as a chemical defense in the privet tree. *Proc. Natl Acad. Sci. USA* 96: 9159–9164. (doi:10.1073/pnas.96.16.9159)

Konno K, Hirayama C, Yasui H, Okada S, Sugimura M, Yukuhiro F, Tamura Y, Hattori M, Shinbo H, Nakamura M. (2010). GABA, β-alanine and glycine in the digestive juice of privet-specialist insects: convergent adaptive traits against plant iridoids. *J. Chem. Ecol*. 36: 983–991. (doi:10.1007/s10886- 010-9842-y)

Koudounas K, Banilas G, Michaelidis C, Demoliou C, Rigas S, Hatzopoulos P. (2015). A defense-related *Olea europaea* β-glucosidase hydrolyses and activates oleuropein into a

potent protein cross-linking agent. *J. Exp. Bot.* 66: 2093–2106. (doi:10.1093/jxb/erv002)

Kounatidis I, Papadopoulos NT, Mavragani-Tsipidou P, Cohen Y, Tertivanidis K. (2008). Effect of elevation on spatio-temporal patterns of olive fruit fly (*Bactrocera oleae*) populations in northern Greece. *J. Appl. Entomol.* 132: 722–33

Kroll JR, Rawel HM, Rohn S. (2003). Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Sci. Technol. Res*. 9: 205–218. (doi:10.3136/fstr. 9.205)

Kubo I, Matsumoto A, Takase I. (1985). A multichemical defense mechanism of bitter olive *Olea europaea* (Oleaceae). *J. Chem. Ecol.* 11: 251–263. (doi:10.1007/BF00988207)

Lauzon CR, McCombs SD, Potter SE, Peabody N. (2009). Establishment and vertical passage of *Enterobacter* (*Pantoea*) *agglomerans* and *Klebsiella pneumoniae* through all life stages of the Mediterranean fruit fly (Diptera: Tephritidae). *Ann. Entomol. Soc. Am*. 102(1): 85-95. http://doi.org/10.1603/008.102.0109

Lauzon CR, Potter SE, Prokopy RJ. (2003). Degradation and detoxification of the dihydrochalcone phloridzin by *Enterobacter agglomerans*, a bacterium associated with the apple pest, *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae). *Environ. Entomol*. 32: 953-962. (doi:10.1603/0046-225X-32.5.953)

Lauzon CR, Sjogren RE, Prokopy RJ. (2000). Enzymatic capabilities of bacteria associated with apple maggot flies: a postulated role in attraction. *J. Chemical Ecol.* 26: 953–967.

Lauzon CR, Sjogren RE, Wright SE, Prokopy RJ. (1998). Attraction of *Rhagoletis pomonella* (Diptera: Tephritidae) flies to odor of bacteria: apparent confinement to specialized members of Enterobacteriaceae. *Environ. Entomol*. 27: 853-857.

Lauzon CR. (2003). Symbiotic relationships of tephritids, p. 115–130. In K. Bourtzis and T. Miller (ed.), Insect symbiosis, vol. 1. *CRC Press*, Boca Raton, FL.

Lemos FJA, TerraWR. (1991). Digestion of bacteria and the role of midgut lysozyme in some insect larvae. *Comp. Biochem. Physiol. B Comp. Biochem*. 100: 265–268. (doi:10.1016/0305-0491(91)90372-K)

Liaropoulos C, Louskas C, Canard M, Laudeho Y. (1977). Release of *Opius concolor* (Hym.: Braconidae) on spring populations of *Dacus oleae* (Dipt., Trypetidae). 1. Experiments in continental Greece. *Entomophaga* 22: 259–64

Liu LJ, Martinez-Sañudo I, Mazzon L, Prabhakar CS, Girolami V, Deng YL, Dai Y, Li ZH. (2016). Bacterial communities associated with invasive populations of *Bactrocera dorsalis* (Diptera : Tephritidae) in China. *Bull. Entomol. Res*. 106(6): 718-728. http://doi.org/10.1017/S0007485316000390

Lumaret R, Ouazzani N, Michaud H, Vivier G, Deguilloux MF, Di Giusto F. (2004). Allozyme variation of oleaster populations (wild olive tree) (*Olea europaea* L.) in the Mediterranean Basin. *Heredity* 92: 343–351.

Lundgren J.G. (2009). Relationships of natural enemies and non-prey foods. Springer, Dordrecht. Manrakhan,

Malorny B, Hoorfar J, Bunge C, Helmuth R. (2003). Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Appl Environ Microbiol*. 69(1): 290-6. (http://pubmedcentral.gov/articlerender.fcgi?artid=152403).

Marchini, D.; Petacchi, R.; Marchi, S. (2017). *Bactrocera oleae* reproductive biology: new evidence on wintering wild populations in olive groves of Tuscany (Italy). *Bull. Insectology*. 70: 121-128.

Margaritopoulos JT, Skavdis G, Kalogiannis N, Nikou D, Morou E. (2008). Efficacy of the pyrethroid alpha-cypermethrin against *Bactrocera oleae* populations from Greece, and improved diagnostic for an iAChE mutation. *Pest Manage. Sci.* 64: 900–8

Margulis L, Fester R. (1991). Symbiosis as a Source of Evolutionary Innovation (Eds). Cambridge, MA: *MIT Press*.

Mari M, Guizzardi M, Brunelli M, Folchi A. (1996). post-harvest biological control of grey mould (*Botrytis cinerea*) on fresh market tomatoes with *Bacillus amyloliquefaciens*. *Crop Protection* 15: 699–705.

Silva FJ, Moret A, Neef A, Belda E. (2008). Bacterial microbiota associated with a *Bactrocera oleae* population from eastern Spain. In First meeting of TEAM, Palma de Mallorca 7–8th

April 2008. Abstract book, Vol. 15 Ed. Miranda-Chueca MA, Universitat de les Illes Balears, Mallor*Ca*.

Mazomenos BE. (1989). *Dacus oleae*. In: Helle W Word Crop Pests (ed) Vol 3A: Fruit Flies: their biology, natural enemies and control. p 169

Mazzini M, Vita G. (1981). Identificazione submicroscopica del meccanismo di trasmissione del batterio simbionte in *Dacus oleae* (Gmelin) (Diptera, Trypetidae). *Redia* 64: 277–318.

Mazzon L, Martinez-Sañudo I, Savio C, Simonato M, Squartini A. (2011). *Stammerula* and other symbiotic bacteria within the fruit flies inhabiting Asteraceae flowerheads. In: Zchori-Fein, E., Bourtzis, K. (Eds.), Manipulative Tenants Bacteria Associated With Arthropods. *CRC Press*, Taylor and Francis Group.

Mazzon L, Martinez-Sañudo I, Simonato M, Squartini A, Savio C, Girolami V. (2010). Phylogenetic relationships between flies of the Tephritinae subfamily (Diptera, Tephritidae) and their symbiotic bacteria. *Mol. Phylogenet. Evol*. 56: 312–326.

Mazzon L, Piscedda A, Simonato M, Martinez-Sanudo I, Squartini A, Girolami V. (2008). Presence of specific symbiotic bacteria in flies of the subfamily Tephritinae (Diptera Tephritidae) and their phylogenetic relationships: proposal of '*Candidatus* Stammerula tephritidis'. *Int. J. Syst. Evol. Microbiol.* 58: 1277–1287. (doi:10.1099/ijs.0.65287-0).

Messina FJ, Jones VP. (1990). Relationship between fruit phenology and infestation by the apple maggot (Diptera: Tephritidae) in Utah. *Ann. Entomol. Soc. Am.* 83: 742–752. (doi:10.1093/aesa/83.4.742)

Min KT, Benzer S. (1997). *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proc. Natl. Acad. Sci. USA* 94: 10792– 10796.

Miranda MA, Miquel M, Terrassa J, Melis N, Monerris M. (2008). Parasitism of *Bactrocera oleae* (Diptera, Tephritidae) by *Psyttalia concolor* (Hymenoptera, Braconidae) in the Balearic Islands (Spain). *J. Appl. Entomol.* 132: 798–805

Miyazaki S, Boush GM, Baerwald RJ. (1968). Amino acid synthesis by *Pseudomonas melophthora* bacterial symbiote of *Rhagoletis pomonella* (Diptera). *J. Insect Physiol*. 14: 513–518.

Monastero R, Delanoue P. (1966). Un grande experimento di lotta biologica artificiale contro la mosca delle olive (*Dacus oleae* Gmel.) a mezzo dell' *Opius conocolor* Sz. *siculus* Mon. in Sicilia (Palmero, luglio-ottobre 1966). *Boll. Ist. Entomol. Agr. Oss Fitopatol.* 6:1–52

Montiel Bueno A, Jones O. (2002) Alternative methods for controlling the olive fly, *Bactrocera oleae*, involving semiochemicals. International Organization for Biological and Integrated Control of Noxious Animals and Plants West Palaearctic Regional Section *(IOBC/WPRS) Bulletin*, 25. 1–11.

Moran NA, Baumann P. (2000). Bacterial endosymbionts in animals. *Curr. Opin. Microbiol*. 3: 270–275.

Moran NA, Degnan PH. (2006). Functional genomics of *Buchnera* and the ecology of aphid hosts. *Mol. Ecol.* 15: 1251-1261.

Moran NA, McCutcheon JP, Nakabachi A. (2008). Genomics and evolution of heritable bacterial symbionts. *Annu. Rev. Genet.* 42: 165-190.

Moran NA, Telang A. (1998). Bacteriocyte-associated symbionts of insects. *Biosci.* 48: 295–304.

Moran NA, Wernegreen JJ. (2000). Lifestyle evolution in symbiotic bacteria: insights from genomics. *Trends Ecol. Evol*. 15: 321–326.

Mourikis PA, Fytizas E. 1970. Review of olive-fly ecology in relation to the sterile-male technique. In Proc. Sterile-Male Technique Control Fruit Flies, pp. 131–39. Vienna: *Int. At. Energy Agency Symp.*

Mouzaki DG, Zarani FE, Margaritis LH. (1991). Structure and morphogenesis of the egg-shell and micropyle apparatus in the olive fly *Dacus oleae* (*Bactrocera oleae*). *J. Insect Morph.* 209: 39-52.

Mouzaki DG, Margaritis LH. (1987). Comparative structural study of the egg-shell (chorion) in *Dacus oleae, Rhagoletis cerasi, Ceratitis capitata* and *Eurytoma amygdali*. In: Economopoulos AP "Fruit Flies": 79-87.

Mukherjee M, Horwitz BA, Sherkhane PD, Hadar R, Mukherjee PK. (2006). A secondary metabolite biosynthesis cluster in *Trichoderma virens*: evidence from analysis of genes

underexpressed in a mutant defective in morphogenesis and antibiotic production. *Curr. Genet.* 50(3): 193-202.

Mukherjee PK, Horwitz BA, Kenerley CM. (2012). Secondary metabolism in Trichoderma – a genomic perspective. *Microbiology* 158(1): 35-45.

Nardi F, Carapelli A, Boore JL, Roderick GK, Dallai R, Frati F. (2010). Domestication of olive fly through a multi-regional host shift to cultivated olives: comparative dating using complete mitochondrial genomes. *Mol. Phylogenet. Evol.* 57(2): 678-686.

Nardi F, Carapelli A, Dallai R, Roderick GK, Frati F. (2005). Population structure and colonization history of the olive fly, *Bactrocera oleae* (Diptera, Tephritidae). *Mol. Ecol.* 14(9): 2729-2738.

Neuenschwander P, Michelakis S, Bigler F. (1981). Abiotic factors affecting mortality of *Dacus oleae* larvae and pupae in the soil. *Entomol. Exp. Appl.* 30: 1–9.

Neuenschwander P, Michelakis S, Holloway P, Berchtold W. (1985). Factors affecting the susceptibility of fruits of different olive varieties to attack by *Dacus oleae* (Gmel.) (Dipt., Tephritidae). *J. Appl. Entomol*. 100: 174–88

Neuenschwander P, Michelakis S. (1978). Infestation of *Dacus oleae* (Gmel.) (Diptera, Tephritidae) at harvest time and its influence on yield and quality of olive oil in Crete. *J. Appl. Entomol.* 86: 420–33

Neuenschwander P, Michelakis S. (1979). Determination of the lower thermal thresholds and day-degree requirements for eggs and larvae of *Dacus oleae* (Gmel.) (Diptera: Tephritidae) under field conditions in Crete, Greece. *Bull. Soc. Entomol. Suisse* 52: 57–74

Neuenschwander P, Michelakis S, Holloway P, Berchtol W. (1985). Factors affecting the susceptibility of fruits of different olive varieties to attack by *Dacus oleae* (Gmel.) (Dipt., Tephritidae). *Z. Angew. Entomol*. 100: 174–188.

Neuenschwander P. (1982). Beneficial insects caught by yellow traps used in mass-trapping of the olive fruit fly, *Dacus oleae*. *Entomol. Exp. Appl*. 32: 286–96

Norrbom AL, Carroll LE, Thompson FC, White IM, Freidberg A. (1999). Status of knowledge. In: Thompson, F.C. (Eds.), Fruit fly expert identification system and systematic information database, Myia 9. IX., pp. 9–48.

Novotny V, Basset Y, Miller SE, Weiblen GD, Bremer B, Cizek L, Drozd P. (2002). Low host specificity of herbivorous insects in a tropical forest. *Nature* 416: 841-844.

Oliver KM, Degnan PH, Burke GR, Moran NA. (2010). Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu. Rev. Entomol*. 55: 247–266.

O'Neill SL, Giordano R, Colbert AME, Karr TL, Robertson HM. (1992). 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci. USA* 89: 2699–2702.

Pais R, Lohs C, Wu Y, Wang J, Aksoy S. (2008). The obligate mutualist *Wigglesworthia glossinidia* influences reproduction, digestion, and immunity processes of its host, the tsetse fly. *Appl. Environ. Microbiol.* 74: 5965–5974.

Pape T, Thompson FC. (2017). Systema Dipterorum. In: Roskov Y., Abucay L., Orrell T., Nicolson D., Bailly N., Kirk P.M., Bourgoin T., DeWalt R.E., Decock W., De Wever A., Nieukerken E. van, Zarucchi J., Penev L., eds.

Paracer S, Ahmadjian V. (2000). Symbiosis: an introduction to biological associations, 2nd ed. *Oxford University Press*, Oxford, United Kingdom.

Pascale A, Vinale F, Manganiello G, Nigro M, Lanzuise S, Ruocco M, Lorito M. (2017). *Trichoderma* and its secondary metabolites improve yield and quality of grapes. *Crop Protection* 92: 176–181. (http://doi.org/10.1016/j.cropro.2016.11.010).

Pavlidi N, Gioti A, Wybouw N, Dermauw W, Ben-Yosef M, Yuval B, Jurkevich E, Kampouraki A, Van Leeuwen T, Vontas J. (2017). Transcriptomic responses of the olive fruit fly *Bactrocera oleae* and its symbiont *Candidatus* Erwinia dacicola to olive feeding. *Nature Sci. Rep*. 7:42633 (http://doi.org/10.1038/srep42633).

Pentzold S, Zagrobelny M, Rook F, Bak S. (2014). How insects overcome two-component plant chemical defense: plantβ-glucosidases as the main target for herbivore adaptation. *Biol. Rev.* 89. 531–551. (doi:10.1111/brv.12066).

Pereira JA, Alves MR, Casal S, Oliveira MBPP. (2004). Effect of olive fruit fly infestation on the quality of olive oil from cultivars Cobrancosa, Madural and Verdeal Transmontana. *Ital. J. Food Sci.* 16:355–65

Petri, L. (1909). Ricerche Sopra i Batteri Intestinali della Mosca Olearia. Roma: Memorie della Regia Stazione di Patologia Vegetale di Roma.

Phillips VT. (1946). The biology and identification of trypetid larvae. *Mem. Am. Entomol. Soc.* 12:1-161.

Prophetou-Athanasiadou DA, Tzanakakis ME, Myroyannis D, Sakas G. (1991). Deterrence of oviposition in *Dacus oleae* by copper hydroxide. *Ent. Exp. Appl*. 61: 1-5.

Raaijmakers JM, Vlami M, de Souza J. (2002). Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek 81. 537–547.

Ragaglini G, Tomassone D, Petacchi R. (2005). Can spring preventive adulticide treatments be assumed to improve *Bactrocera oleae* (Rossi) management? *IOBC/WPRS Bull*, 30: 309–314.

Ras E, Beukeboom LW, Càceres C, Bourtzis K. (2017). Review of the role of gut microbiota in mass rearing of the olive fruit fly, *Bactrocera oleae*, and its parasitoids. Entomol. Exp. Appl. 1–20. (DOI: 10.1111/eea.12609).

Raspi A, Canale A, Felicioli A. (1997). Relationship between the photoperiod and the presence of mature eggs in *Bactrocera oleae* (Gmel.) (Dipthera Tephritidae). *IOCB/WPRS Bull.* 20: 46–54.

Raspi A, Iacono E, Canale A. (2002). Variable photoperiod and presence of mature eggs in olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera Thephritidae). *Redia*, LXXXV: 111– 119.

Raspi A, Loni A. (1994). Alcune note sull'allevamento di *Opius concolor* (Szèpl.) (Hymenoptera: Braconidae) e su recenti tentativi d'introduzione della specie in Toscana ed in Liguria. *Frustula Entomol*. 17: 135-145.

RattanapunW, AmornsakW, Clarke AR. (2009). *Bactrocera dorsalis* preference for and performance on two mango varieties at three stages of ripeness. *Entomol. Exp. Appl*. 131: 243–253

Ratzka C, Gross R, Feldhaar H. (2012). Endosymbiont Tolerance and Control within Insect Hosts. *Insects*, 3. 553–572. (http://doi.org/10.3390/insects3020553).

Rice RE, Phillips PA, Stewart-Leslie J, Sibbett GS. (2003). Olive fruit fly populations measured in central and southern California. *California Agri.* 57: 122–127.

Rice RE. (1999). Olive fruit fly, *Bactrocera* (*Dacus*) *oleae*. *UC Plant Protection Quarterly*, 9: 2.

Riegler M, Stauffer C. (2002). *Wolbachia* infections and superinfections in cytoplasmically incompatible populations of the European cherry fruit fly *Rhagoletis cerasi* (Diptera, Tephritidae). *Mol. Ecol*. 11: 2425–2434.

Rigaud T. (1997). Inherited microorganisms and sex determination of arthropod hosts. In: Influential passengers: inherited microorganisms and arthropod reproduction. Ed. by O'Neill SL, Hoffmann AA, Werren JH, *Oxford University Press*, Oxford, UK, 81–101.

Rizzo R, Caleca V. (2006). Resistance to the attack of *Bactrocera oleae* (Gmelin) of some Sicilian olive cultivars. Proceedings of Olivebioteq 2006. Second International Seminar Biotechnology and quality of olive tree products around the Mediterranean Basin^{o} November 5th-10th, Mazara del Vallo, Marsala, Italy; 2006.

Robacker DC, Lauzon CR. (2002). Purine metabolizing capability of *Enterobacter agglomerans* affects volatiles production and attractiveness to Mexican fruit fly. *J. Chem. Ecol*. 28: 1549–1563.

Rocha LS, Mascarenhas RO, Perondini ALP, Selivon D. (2005). Occurrence of *Wolbachia* in Brazilian samples of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). *Neotrop Entomol* 34(6): 1013–1015

Rosi MC, Sacchetti P, Librandi M, Belcari A. (2007). Effectiveness of different copper products against the olive fly in organic olive groves. *Integr. Prot. Olive Crops*. *30*(9): 277–281.

Ruiz Castro A. (1948). Fauna Entomologica del Olivo en España. Instituto Español de Entomologia, Madrid.

Russell JA, Latorre A, Sabater-Munoz B, Moya A, Moran NA. (2003). Side-stepping secondary symbionts: Widespread horizontal transfer across and beyond the Aphidoidea. *Mol. Ecol*. 12: 1061–1075.

Sacchetti P, Belcari A, Del Pianta R. (2004). Utilizzo di prodotti ad azione antibatterica per il controllo della mosca delle olive. In: La difesa dai fitofagi in condizioni di olivicoltura biologica, Atti Accademia nazionale dell'Olivo e dell'Olio, Spoleto, 29-30 October 2002: 23-33.

Sacchetti P, Granchietti A, Landini S, Viti C, Giovannetti L, Belcari A. (2008). Relationships between the olive fly and bacteria. *J. Appl. Entomol*. 132: 682–689.

Saour G, Makee H. (2004). A kaolin-based particle film for suppression of the olive fruit fly Bactrocera oleae Gmelin (Dip., Tephritidae) in olive groves. J. Appl. Entomol. 128:28–31

Salwa Sholla M, Metwally Kottb R. (2017). Bioactivity of *Trichoderma* (6-Pentyl α-pyrone) against *Tetranychus urticae* Koch (Acari: Tetranychidae). *Egypt. Acad. J. Biolog. Sci*. 10(3): 29–34

Saridaki A, Bourtzis K. (2010). Wolbachia: more than just a bug in insects genitals. *Curr. Opin. Microbiol.* 13: 67–72.

Savio C, Mazzon L, Martinez-Sanudo I, Simonato M, Squartini A, Girolami V. (2012). Evidence of two lineages of the symbiont '*Candidatus* Erwinia dacicola' in Italian populations of *Bactrocera oleae* (Rossi) based on 16S rRNA gene sequences. *Int. J. Syst. Evol. Micrbiol*. 62: 179– 187.

Schoonhoven LM, van Loon JJA, Dicke M. (2005). Insect-plant biology (2nd ed.). Oxford: *Oxford University Press.*

Selivon D, Perondini ALP, Ribeiro AF, Marino CL, Lima MMA, Coscrato VE. (2002). *Wolbachia* endosymbiont in a species of the *Anastrepha fraterculus* complex (Diptera, Tephritidae). *Invert. Reprod. Develop*. 42: 121-127.

Shikano I, Cory JS. (2014). Dietary mechanism behind the costs associated with resistance to *Bacillus thuringiensis* in the cabbage looper, Trichoplusiani. *PLoS ONE, 9*(8). https://doi.org/10.1371/journal.pone.0105864

Sholberg, P.L., Marchi, A. and Bechard, J. (1995). Biocontrol of post-harvest diseases of apple using *Bacillus* spp. isolated from stored apples. *Can. J. Microbiol*. 41: 247–252.

Sivasithamparam K, Ghisalberti EL. (1998). Secondary metabolism in *Trichoderma* and *Gliocladium*. In: Trichoderma and Glioclaudium, Kubicek, C.P.; Harman, G.E., Eds., Taylor and Francis: London. Vol 1. pp. 139–205.

Skouras PJ, Margaritopoulos JT, Seraphides NA, Ioannides IM, Kakani EG. (2007). Organophosphate resistance in olive fruit fly, *Bactrocera oleae*, populations in Greece and Cyprus. *Pest Manage. Sci.* 63: 42–48.

Slansky FJ, Rodriguez JG. (1987). Nutritional ecology of insects, mites, spiders, and related invertebrates. New-York: John Wiley.

Smith AH, Zoetendal E, Mackie RI. (2005). Bacterial mechanisms to overcome inhibitory effects of dietary tannins. *Microb. Ecol.* 50: 197–205. (doi:10.1007/s00248-004-0180-x)

Spadafora A, Mazzuca S, Chiappetta FF, Parise A, Perri E, Innocenti AM. (2008). Oleuropein-specific-β-glucosidase activity marks the early response of olive fruits (*Olea europaea*) to mimed insect attack. *Agric. Sci. China* 7: 703–712. (doi:10.1016/S1671-2927(08)60105-4)

Stammer HJ. (1929). Die bakterien symbiose der trypetiden (Diptera). *Zoomorphology.* 15: 481–523.

Stasinakis P, Katsares V, Mavragani-Tsipidou P. (2001). Organophosphate resistance and allelic frequencies of esterases in the olive fruit fly *Bactrocera oleae* (Diptera: Tephritidae). *J. Agric. Urban Entomol.* 18: 157–68

Stouthamer R, Breeuwer JA, Hurst GD. (1999). *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol*. 53: 71–102.

Stouthamer R, Luck RF, Hamilton WD. (1990). Antibiotics cause parthenogenetic *Trichogramma* to revert to sex. *Proc. Natl. Acad. Sci. USA* 87. 2424–2427.

Sun X, Cui L, Li Z. (2007). Diversity and Phylogeny of *Wolbachia* infecting *Bactrocera dorsalis* (Diptera: Tephritidae) populations from China. *Environ. Entomol*. 36: 1283–1289.

Taiz L, Zeiger E. (2010). Secondary metabolites and plant defense. In Plant physiology (eds L Taiz, E Zeiger), pp. 369–400. Sunderland, MA: Sinauer Associates Inc.

Tamendjari A, Angerosa F, Bellal MM. (2004). Influence of *Bactrocera oleae* infestation on olive oil quality during ripening of Chemlal olives. *Ital. J. Food Sci.* 16: 343–54

Terra WR, Ferreira CL. (2012). Molecular and evolutionary physiology of insect digestion. In Insect bioecology and nutrition for integrated pest management (eds AR Panizzi, JRP Parra), pp.93–119. BocaRaton, FL: *CRCPress*.

Hort A, Theophrastus. (1916). Enquiry to Plants and Minor Works on Odours and Weather Signs: In Two Volumes. Cambridge (Mass.): *Harvard UP Print*.

Thomas DB, Mangan RL. (2005). Nontarget impact of spinosad GF-120 bait sprays for control of the Mexican fruit fly (Diptera: Tephritidae) in Texas citrus. *J. Econ. Entomol.* 98: 1950–56

Torres-Villa LM, Rodriguez-Molina MC, Martinez JA. (2003). Olive fruit fly damage and olive storage effects on paste microflora and virgin olive oil acidity. *Grasas Aceites* 54: 285–94

Tremblay E. (1960). Ciclo cromosomico e simbiosi endocellulare nella *Diaspis pentagona* Targ. - *Boll. Lab. Ent. Agr.* F. Silvestri Portici 18: 49-228.

Tremblay E. (1994). Entomologia Applicata. III. *Liguori Editore*. 2: 132-135.

Tsahar E, del Rio CM, Izhaki I, Arad Z. (2005). Can birds be ammonotelic? Nitrogen balance and excretion in two frugivores. *J. Exp. Biol*. 208: 1025–1034.

Tsakas S, Krimbas CB. (1970). The genetics of *Dacus oleae*. IV. Relation between adult esterase genotypes and survival to organophosphate insecticides. *Evolution* 24: 807–15

Tsiropoulos GJ. (1977). Reproduction and survival of adult *Dacus oleae* feeding on pollens and honeydews. *Environ. Entomol*. 6: 390–392.

Tsiropoulos GJ. (1980). Major nutritional requirements of adult *Dacus oleae (*Diptera, Tephritidae). *Ann. Entomol. Soc. Am*. 73: 251–253.

Tsiropoulos GJ. (1983). The importance of dietary amino acids on the reproduction and longevity of adult *Dacus oleae* (Gmelin) (Diptera Tephritidae). *Arch. Int. Physiol. Biochim. Biophys.* 91: 159–164.

Tsiropoulos GJ. (1984). Amino-acid synthesis in adult *Dacus oleae* (Gmelin) (Diptera Tephritidae) determined with [U-14C] glucose. *Arch. Int. Physiol. Biochim*. 92: 313–316.

Tsitsipis JA. (1977). Effect of constant temperatures on eggs of olive fruit fly, *Dacus oleae* (Diptera: Tephritidae). *Ann. Zool. Ecol. Anim*. 9: 133–39

Tsitsipis JA. (1980). Effect of constant temperatures on larval and pupal development of olive fruit flies reared on artificial diet. *Environ. Entomol.* 9: 764–68

Tzanakakis ME. (1985). Considerations on the possible usefulness of olive fruit fly symbionticides in integrated control in olive groves. In: Cavalloro R. and Crovetti A. (eds.): Integrated Pest Control in Olive-Groves. Proc. CEC/FAO/IOBC. International Joint Meeting, Pisa, 3-6 April 1984: 386-393.

Tzanakakis ME. (2003). Seasonal development and dormancy of insects and mites feeding on olive: a review. *Neth. J. Zool*. 52: 87–224

Tzanakakis ME. (2006). Insects and Mites Feeding on Olive: Distribution, Importance, Habits, Seasonal Development and Dormancy. Leiden: *Brill Acad. Publ*. 182 pp.

Tzanakakis ME. (2006). Insects and Mites Feeding on Olive: Distribution, Importance, Habits, Seasonal Development, and Dormancy. Brill, Leiden, Boston.

van Tets IG, Korine C, Roxburgh L, Pinshow B. (2001). Changes in the composition of the urine of yellow-vented Bulbuls (*Pycnonotus xanthopygos*): the effects of ambient temperature, nitrogen, and water intake. *Physiol. Biochem. Zool*. 74: 853–857.

Viale E, Martinez-Sañudo I, Brownb JM, Simonato M, Girolami V, Squartini A, Bressan A, Faccoli M, Mazzon L. (2015). Pattern of association between endemic Hawaiian fruit flies (Diptera, Tephritidae) and their symbiotic bacteria: Evidence of cospeciation events and

proposal of "*Candidatus* Stammerula trupaneae". *Mol. Phylogenet. Evol.* 90: 67–79. (http://doi.org/10.1016/j.ympev.2015.04.025).

Vinale F, Marra R, Scala F, Ghisalberti EL, Lorito M, Sivasithamparam K. (2006). Major secondary metabolites produced by two commercial *Trichoderma* strains active against different phytopathogens. *Lett. Appl. Microbiol*. 43(2): 143-8.

Vinale F, Sivasithamparam K, Ghisalberti EL, Woo SL, Nigro M, Marra R, Lorito M. (2014). *Trichoderma* secondary metabolites active on plants and fungal pathogens. *Open Mycol. J. 8*(1): 127–139. https://doi.org/10.2174/1874437001408010127

Wackers FL. (2005). Suitability of (extra-) floral nectar, pollen, and honeydew as insect food sources. In: Plant-Provided Food for Carnivorous Insects: Protective Mutualism and Its Applications (F.L. Wackers, P.C.J. Rijn and J. Bruin, eds), pp. 17–74. *Cambridge University Press*, New York.

Wang XG, Johnson MW, Daane KM, Nadel H. (2009). High summer temperatures affect the survival and reproduction of olive fruit fly (Diptera: Tephritidae). *Environ. Entomol*. 38: 1496-1504.

Wernegreen JJ. (2002). Genome evolution in bacterial endosymbionts of insects. *Nature Rev. Genet*. 3: 850-861.

Werren JH, Zhang W, Guo LR. (1995). Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. *Proc. R. Soc. B* 261: 55–63.

Werren JH. (1997). Biology of *Wolbachia*. *Annu. Rev. Entomol*. 42: 587–609

Wharton RA. (1989). Classical biological control of fruit-infesting Tephritidae. InWorld Crop Pests: Fruit Flies—Their Biology, Natural Enemies and Control, ed. AS Robinson, G Hooper, 3B:303–13. Amsterdam: Elsevie

White IM, Elson-Harris MM. (1992). Fruit Flies of Economic Significance: Their Identification and Bionomics. Wallingford, USA: CAB International.

White IM. (2006). Taxonomy of the Dacina (Diptera: Tephritidae) of Africa and the Middle East. *Afr. Entomol. Mem.* pp. 152.

Whitehead SR, Bowers MD. (2013). Evidence for the adaptive significance of secondary compounds in vertebrate-dispersed fruits. *Am. Nat.* 182: 563–577. (doi:10.1086/673258)

Yokoyama VY, RendonPA, Sivinsk J. (2008). *Psyttalia* cf. *concolor* (Hymenoptera: Braconidae) for biological control of olive fruit fly (Diptera: Tephritidae) in California. *Environ. Entomol*. 37: 764–773

Zabalou S, Apostolaki A, Livadaras I, Franz G, Robinson AS, Savakis C, Bourtzis K. (2009). Incompatible insect technique: incompatible males from a *Ceratitis capitata* genetic sexing strain. *Entomol. Exp. Appl.* 132: 232–240.

Zabalou S, Riegler M, Theodorakopoulou M, Stauffer C, Savakis C, Bourtzis K, (2004). Wolbachia-induced CI as a means for insect pest population control. *Proc. Natl. Acad. Sci. USA* 101: 15042–15045.

Zamora R, Alaiz M, Hidalgo FJ. (2001). Influence of cultivar and fruit ripening on olive (Olea europaea) fruit protein content, composition, and antioxidant activity. *J. Agric. Food Chem.* 49: 4267–4270. (doi:10.1021/jf0104634)

Zervas GA. (1982). Reproductive physiology of *Dacus oleae* (Gmel.) (Diptera: Trypetidae). Comparison of a wild and artificially reared flies. *Geoponica* 282: 10–14.

Zientz E, Dandekar T, Gross R. (2004). Metabolic interdependence of obligate intracellular bacteria and their insect hosts. *Microbiol. Mol. Biol. Rev.* 68: 745–770.

Zohary D, Hopf M. (2000). Domestication of Plants in the Old World: the Origin and Spread of Cultivated Plants. *Oxford University Press.*

Zohary D, Spiegel-Roy P. (1975). Beginnings of fruit growing in the Old World. *Science* 187: 319–327.

Zohary D. (1994). The wild genetic resources of the cultivated olive. *Acta Horticulturae,* 365: 62–65.

Zouros E, Krimbas CB. (1970). Frequency of male bigamy in natural population of the olive fruit fly *Dacus oleae* as found by using enzyme polymorphism. Entomol. Exp. Appl. 13: 1–9.

Martinez-Sañudo, I. (2009). Phylogenetic studies of tephritid flies (Diptera, Tephritidae) and their symbiotic bacteria. Ph.D thesis. University of Padova, Italy.

Fytizas E, Tzanakakis ME. (1966). Some effects of Streptomycin when added to the adult food, on the adults of *Dacus oleae* (Diptera: Tephritidae) and their progeny. *Ann. Entomol. Soc. Am.* 59: 269–273.