Behavioural Resistance in Insects: Its Potential Use as Bio Indicator of Organic Agriculture

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Abstract. Most of the investigations carried out on the resistance of insects to pesticides have been focused on the physiological and biochemical mechanisms. However, the behavioural answers that pesticide induces in the insects have received very little attention. The symptoms from getting pesticides on the cuticular surface of insects, as neurotoxical pre mortem effects, include spasmodic activity, hyperactivity and leak of the surfaces impregnated by the pesticides. These reactions provide a first barrier of defense, named behavioural resistance. Previous experiments carried out on olive groves usually subjected to pesticide application have allowed to visualize a reaction of leak of the natural enemies from treated areas, which is reflected as an increase of its rate of capture in sticky chromatic traps, in relation to the free of pesticides areas, on olive groves, usually subjected to pesticide application. The aim of this investigation is to evaluate the reaction of these insects’ species under different agricultural pest management: i- A conventional olive grove, where pesticides are usually applied; and ii- An organic olive grove, where pest management depends exclusively on the role of the natural enemies. During the spring of 2016, experimental applications have been carried out in two olive groves of the province of Jaén (south of Spain) by means of a commercial pesticide application, in order to evaluate the reactions induced in the main species of olive predators. Six pairs of plots were randomly selected in both conventional and organic olive groves, three of these were pesticide sprayed, whereas a second series of three plots were free of pesticide application. Sticky yellow traps were installed in both treated and control plots just after application of pesticide. The results allow to determine the existence of two different reactions of the predators in both types of olive groves. In the plots of the conventional management, a significant increase of the capture values was observed. However, in the ecological groves, the same insects’ species showed a lack of behavioural resistance. The ecological implications of this study open a new field of research, which provides new criteria to assess the qualification of the organic agriculture.

1. Introduction
The appearance of resistance of the insects to the pesticides is one of the most notable and catastrophic consequences of the chemical systematic fight, which has focused the attention of numerous investigations [1, 2, 3, 4, 5, 6, 7]. Most of them have centred on the biochemical and / or physiological mechanisms that pesticides cause in insects [8], and little attention has been devoted to the behavioural answers. The appearance of behavioural responses induced by the application of pesticides has been firstly reported by Carrero [9], by Varela and González-Ruiz [10] and González-Ruiz [11] in
communities of natural enemies of the olive pests; in maize culture [12], cabbage [13], as well as in insects vectors of human diseases [14; 15; 16; 17; 18].

The behavioural resistance can be defined as the set of behavioural modifications induced in the individuals affected by the pesticides, in respect of their normal behaviour, which provides the first barrier or mechanism of detoxification to them. The affected insects having this skill show a trend to flight towards free zones of pesticide, avoiding the contacts with the sprayed surfaces [9]. The reaction of flight towards free zones of pesticide allows them to avoid the possibility of new contacts with the toxin and to try to eliminate the residues from the cuticular affected zones. In this respect, the pre mortem symptoms would be included here, as described on certain species of dipteran affected by the pesticides: spasmodic movements, overexcitement, and hyperactivity [17; 18; 19; 20].

The behavioural resistance has been described in dipteran affected by organochlorides, organophosphates, carbamates and piretroids [21]. Due to its importance, it has been taken into account in the integrated programs of pest control [12; 22; 23]. The induced escape reaction has been a useful tool for the evaluation of the toxicity of different types of chemical insecticides on the entomofauna of olives subjected to conventional management [24]. Since hyperactivity is induced in those species that exhibit the capacity to develop behavioural resistance, this implies an increase in being intercepted by the sticky, pesticide-free traps. As a final result, in a zone of the crop treated with pesticides, the species that show behavioural resistance shows a greater number of catches in the sticky traps in pesticide treated areas in relation to control ones. This is explained by the fact that, in the traps of the treated areas, the effect of the chromatic attractiveness is added to that produced by the disturbance of the chemical treatment.

However, since the studies carried out on behavioural resistance come from crops subjected to the systematic application of chemical control, the objective of this work is to evaluate the reaction of the natural enemies in organic ecosystems, in which chemical control is never applied and pest control depends almost exclusively on the entomophagous activity of natural enemies.

2. Material and Methods
2.1. Experimental set up.
The study was carried out in olive groves of the province of Jaén (Andalusia, southern Spain) during the spring and summer of 2016. Two models of olive groves have been selected:

*Conventional agricultural management.* It has coordinates 37° 36´18.20´´ N 3º28´33.59´´ W, the olive trees are of the picual variety, 20-30 years old, planted at a density of 100 olive / hectare. Preemergence herbicides are applied to keep it free from adventitious plant cover. Insect pests are controlled by the application of phytosanitary treatments based on commercial organophosphate insecticides, on a scheduled basis. Two insect pests, the olive moth, Prays oleae Bern. (Lep., Praydidae) and the olive fly, Bactrocera oleae Gmell (Dip., Tephritidae) are the main targets of the pesticide applications in south Spain olive groves. To prevent the action of these pests, dimethoate 40% (400 g / l) (BASF) is usually applied, at a concentration of 0.1% (100 cc / hl), which takes place during the spring and autumn, respectively.

*Organic olive grove.* The selected olive grove has the coordinates 37° 37´24.38´´ N 3º 29´51.22´´ W. No kind of synthetic chemical is applied in this olive grove, including pesticides and fertilizers. This olive grove has an adventitious herbaceous cover during autumn, winter and spring. Pest control depends almost exclusively on the activity of natural enemies.

To monitor the reaction displayed by the species of the predatory fauna is the main objective of our study. During the spring, one of the two most significant pesticide applications are usually carried out
in the olive groves. Its target is the anthophagous generation of the olive moth, *Prays oleae*. Then, experimental pesticide application was carried on May 7, 2016 during phenological stage of olives FI (beginning of the opening of flower buds), when the percentage of open flowers was 5% - 10%. In both olive groves (traditional and organic), three pairs of plots of 30 m x 30 m (900 m²) have been randomly established, each containing 16 olive trees, and spaced more than 100 m apart. For the application of the experimental insecticide treatment, 3 of the plots in each of the two olive groves (conventional and organic) were randomly selected, while the remaining 3 plots of each olive grove have been considered control plots. The insecticide was dimethoate 40% (400 g / l) (BASF), at a concentration of 0.1% (v / v), sprayed by a backpack equipment (MATABI Evolution 16) of 16 l capacity. The average solution volume applied was 3-5 l per tree, the air temperature during the spray was 15-18 °C and in calm conditions. The olives of the remaining 3 plots of each olive grove, corresponding to the control plots, were sprayed with water.

2.2. Samplings.

Just after the pesticide application, chromatic sticky traps were installed in the olive trees of each of the six plots, both in the traditional olive grove as in the organic. The traps, of 20 cm x 14 cm dimensions and yellow color, were located in the S-E sector of the olive trees, at a height of 1.70 cm from the ground. One unit was installed in each tree, then the number of traps, per sampling interval, was 16 units / plot; 48 units / type of olive grove and a total of 96 units for each sampling.

The traps have been renewed at intervals of 5 days, so until the end of the study (end of June), there have been four samplings: June, 7-12; June, 12-17; June, 17-22 and June, 22 -27. In order to minimize the effect of the pseudo-replication, at every sampling two pairs of plots (one from control group and one from the sprayed group) were randomly selected and 6 traps from every plot were randomly selected, too. After being removed, the sticky traps were examined in laboratory by means of magnifying glass binoculars, where the taxonomical determination was achieved and the number of individuals per predator species was registered.

The statistical analysis was carried out using the statistical package Statistica [13.0/ Sept.2015]. Initially, the normality of the distributions was verified by Chi-square tests and the Kolmogorov-Smirnov test (K-S), with the Liliefors correction, and the Levene test to evaluate the homogeneity of the variances for a variable calculated for two or more groups. For the non-parametric distributions Kruskal-Wallis (K-W) analysis of the variance has been applied. The Mann-Whitney U test (M-W) was applied for the comparison between two groups. For the interpretation of the results, the methodology developed by Varela and González-Ruiz [24], has been taken into account.

3. Results and discussions

3.1 Species of predators caught.

A total number of 4439 specimens, belonging to 10 species of predators, have been identified in the sticky traps, which correspond to the orders Thysanoptera, Neuroptera, Hemiptera, Raphidioptera, Coleoptera and Diptera.

The most abundant predator (85%) was *Aeolothrips intermedius* Bagnall (Thys., Aeolothripidae). This predator is present in a wide range of cultures [25; 26], attacking to several thysanopteran species preferably, as well as to phytophagous mites [25]. In the olive grove, its potential preys are represented by *Liothrips oleae* Costa (Thys., Phlaeotripidae) (Arambourg, 1986), and specially by the mites *Aceria oleae* Nalepa and *Oxycenus maxelli* Keifer (Acari, Eriophyidae).

The neuropteroids reached a proportion of 5.4% of the total catches. Among them, the most abundant was *Chrysoperla agilis* (Neur., Chrysopidae) and the snakefly *Harraphidia laufferi* (Navás, 1915) (Raph., Raphidiidae). The chrysopids, referred to as *Chrysoperla carnea* (Stephens, 1836) sensu
lato, the so-called “common green lacewings” belong to the best tested beneficial insects regarding their pesticide susceptibility [27]. The taxonomic status of the species in question has been changing, and instead of a particular species, a complex of sibling or cryptic species, the Chrysoperla carnea complex, or carnea-group [28; 29], should be taken into account. The existence of various sibling species is assumed: 1) Ch. affinis former Ch. kolthoffi [28]; 2) Chrysoperla lucasina (Lacroix, 1912) [29]; 3) Chrysoperla carnea sensu stricto [28] or Chrysoperla pallida [30] and 4) Chrysoperla agilis sp. nov. [31]. The studies carried out in the south of Spain showed the presence of these cryptic species in Andalusian olive groves, of which Ch. agilis was dominant (>90 %), [32], in agree with the results of this study. In the olive groves, the carnea-complex are polyphagous and effective predators in the natural control of the olive moth Prays oleae [32; 33], hemipterans psyllids, Euphyllura olivina, and scales species of families Coccidae and Diaspididae.

The snakefly Harraphidia laufferi (Raph., Raphidiidae) is frequently present in the olive groves [34], preying on bark beetles larvae of Phloeotribus scarabaeoides Bern. (Col., Scolytinae) [35] at the larval stage. According to larval ecology, they must be relevant natural enemies of the olive borer, Euzophera pinguis (Lep., Pyralidae) [34]. At the adult stage, raphidiopteran are also predators, feeding on several sap suckers species of the herbaceous cover (Hem., Aphidiidae) as well as on nymphs of olive psyllid, Euphyllura olivina [34].

The hemipteran species of predators reached a proportion of ca. 6% of the total catches. Anthocorids bugs have been commonly cited as predators present in olive orchards [36; 37; 38]. The species collected in this study were Anthocoris nemoralis F., the pirate bug, Orius laevigatus (Fieber), and Temnostethus pusillus (Horváth). They are common in a wide range of crops and both, adults and nymphs, are general predators which consume a variety of pests including mites, thrips, aphids, small caterpillars, psyllids and whiteflies. In the olive three, they feed on psyllids, Euphyllura olivina (Hem., Psyllidae) spider mites [36], thrips, Liothrips oleae [33; 37] and small caterpillars, Prays oleae [33].

In a lower proportion (2,3%), ladybirds (Col., Coccinellidae) were also caught on the sticky traps. The collected species were Stethorus punctillum Weise, Adalia bipunctata L. and Coccinella septempunctata L. Former species, known as the spider mite destroyer lady beetle, is an effective agent in IPM programs, since both adults and larvae feed on mites [39]. It occurs in tree plantations, gardens and crop fields where it mainly attacks two-spotted spider mite (Tetranychus urticae Koch. Ladybirds C. septempunctata are predators of the several major scales pests, such as the black scale, Saissetia oleae Oliv. (Hem., Coccidae), the olive scale, Parlatoria oleae Colvée, the oystershell scale, Lepidosaphes ulmi L. [33], and the oleander scale, Aspidiotus nerii Bouché (Diaspididae) [36].

A lower proportion of the catches (0,7 %) corresponded to the dipteral predators: Syrphus ribesii L. and Sphaerophoria scripta L. (Syrphidae). In the olive groves, the hoverflies larvae have been reported as predators of the olive psyllid, Euphyllura olivina [25; 36].

3.2 Influence of the agricultural management on predators abundance.
To analyze the influence of the agricultural management practices (traditional and organic) on the abundance of predators, control plots are considered here. As shown in Figures 1 to 6, predators presented, in all cases, a higher abundance in the organic olive grove than in the traditional one (M-W test, U=83, p<0,0001 to Aeolothrips intermedius; U=6,5, p<0,0001 to Chrysoperla agilis; U=132, p<0,001 to Harraphidia laufferi; U=6,5 p<0,05 to Orius laevigatus and U=5, p<0,05 to Anthocoris nemoralis and U=27, p<0,001 to Coccinella septempunctata).

Reducing the abundance of natural enemies is an obvious consequence of the regular application of pesticides in agroecosystems [26; 38]. It has been reported to Aeolothrips intermedius, Coccinella septempunctata [26] and to anthocorids [38]. As it has been reported, the common green lacewings
have usually displayed the ability to develop physiological resistance to pesticides [40; 27]. In spite of this, our results show a significant reduction of the abundance of natural enemies in the ecosystem subjected to a regular application of pesticides.

The difference in abundance between the two types of olive growing compared here, is especially evident in the snakely Harraphidia laufferi, which has been collected only in organic olive orchard. Since its larvae require from 2 to 3 years for their complete development [34] this makes it a particularly vulnerable species, then their relatively scarcity among predators may be a consequence of their greater susceptibility to pesticide applications.

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**Figures 1, 2, 3, 4, 5 6.** Statistical parameters (median, maximum, minimum and interval Q25-Q75) correspond to the predators collected in the sticky traps of control plots in both conventional and organic management. Aeolothrips intermedius (Fig. 1), Chrysoperla agilis (Fig. 2), Orius laevigatus (Fig. 3), Harraphidia laufferi (Fig. 4), Anthocoris nemoralis (Fig. 5) and Coccinella septempunctata (Fig. 6). Asterisks indicate statistical significance [Mann-Whitney test, p<0,05 (*); p<0,01 (**), p<0,001 (***)].

### 3.3 Experimental insecticide treatments: Induction of behavioral resistance.

The results of the experimental application of the insecticide indicate that two opposite situations occurs in the two types of management considered.

**i- Conventional management.** The capture level of predators in traps of the sprayed plots is greater than in the control plots (Figures 7 to 12). This increase is more evident in the more abundant predators, and it is therefore statistically significant for Aeolothrips intermedius (Fig. 7, M-W test, U=193, p<0,05) and Ch. agilis (Fig. 8, M-W test, U=23, p<0,005). The level of catches of Orius laevigatus (Fig. 9), Anthocoris nemoralis (Fig. 11) and species of Coccinellidae (Fig. 12) were also slightly greater in the sprayed plots, although in those cases was not statistically significant (one way ANOVA, p>0,05). These results are in line with what has been observed in previous studies, so that this increase in the treated plots of the conventional olive grove is attributed to the escape reaction to
pesticide-free zones by insects with behavioral resistance [24]. The escape reaction and the increase of insects activity after the application of pesticides, have been reported in the parasite *Cimex lectularius* [15]. This may occur in most of the predators studied here (except for *H. laufferi* and probably *A. nemoralis*), having a relatively high ability to develop behavioral resistance. It seems that color sticky traps are not appropriate to monitorize the populations of natural enemies in conventional crops, since the results would provide a wrong idea, as a result of the effect of the behavioural resistance. It is especially important to take it into account in IPM programs, as reported in *Sitophilus zeamais* [12].

**Figures 7, 8, 9, 10, 11, 12.** Statistical parameters (median, maximum, minimum and interval Q25-Q75) correspond to the predators collected in the sticky traps of the dimethoate-sprayed plots (gray boxes) and control plots (white boxes) in both conventional and organic management to *Aeolothrips intermedius* (Fig. 8), *Chrysoperla agilis* (Fig. 8), *Orius laevigatus* (Fig. 9), *Harraphidia laufferi* (Fig. 10), *Anthocoris nemoralis* (Fig. 11) and *Coccinella septempunctata* (Fig. 12). Asterisks indicate statistical significance [Mann-Whitney test, p<0,05 (*); p<0,01 (**), p<0,001. (***)].

Our results confirm that certain predators, including *Ch. agilis*, have great potential to develop defense mechanisms against insecticides, in agreement with the established on several species of the *carnea*-complex [27; 40; 41].

**ii- Organic management.** Unlike the previous one, in the sprayed plots of the organic grove, the number of catches showed a decrease with respect to the control plots. This decrease was statistically significant to *Aeolothrips intermedius* (Fig. 7; M-W test, U=168,5; p<0,01), *Ch. agilis* (Fig. 8, M-W test, U=28, p<0,01), *Orius laevigatus* (Figure 9; M-W test, U=11,5; p<0,001), *Harraphidia laufferi* (Fig. 10; M-W test, U=162; p<0,001) and *Anthocoris nemoralis* (Figure 11; M-W test, U=5,5; p<0,05). Number of catches was also lower to *Coccinellidae* predators (Fig. 12), although it was not statistically significant (M-W test; p>0,05).

Therefore, two completely different behaviors are shown for the same species of predator, such as *Ch. agilis* and *A. intermedius*, depending on the agricultural management (Figs. 6 and 7), which corresponds very probably to their different degree of susceptibility to pesticides. The existence of a
wide range of pesticide resistance has been reported in chrysopidae predators [42], where the resistance varies according to the geographical origin of lacewings. This could be very probably due to the agricultural management practices applied and therefore to the selection pressure exerted in the original ecosystem.

Figures 13 to 18 show the variation of the catch values of three species in the different sampling intervals, in treated and control plots of both types of olive groves. In the conventional olive grove the effect induced by the application of the insecticide is maintained throughout the whole period. Capture values were higher in the sprayed plots for *Aeolothrips intermedius* (Fig. 13), *Chrysoperla agilis* (Fig. 15) and *Orius laevigatus* (Fig. 17). In the latter species the effect begins to be observed somewhat later due to the absence of adults in the biotopes during the first sampling period. Parallel to the above, in the organic olive grove, the decrease of catches for these predators is maintained throughout the study period in sprayed plots. Moreover, in the case of *Orius laevigatus* (Fig. 16) and *Chrysoperla agilis* (Fig. 18) the catch records between sprayed and control plots shows their maximum difference in the last two sampling periods.

Figures 13, 14, 15, 16, 17, 18. Statistical parameters (median, maximum, minimum and interval Q25-Q75) correspond to the predators *Aeolothrips intermedius* (Figs. 13 and 14); *Chrysoperla agilis* (Figs. 15 and 16), and *Orius laevigatus* (Figs. 17 and 18). Data from the chromatic traps of the dimethoate sprayed (gray boxes) and control (white boxes) plots of both types of crop management, in the four sampling intervals performed (June, 7-12; June, 12-17; June, 17-22 and June, 22-27).

The selection pressure caused by the pesticides therefore determines the selective survival of those best adapted lineages through the selection of resistance mechanisms over generations, which would explain the remarkable variation observed in the two biotopes studied here. This agrees with what has been reported on *Plutella xylostella* in cabbage [13] where it is established that under a strong selection pressure, a population is more susceptible to develop behavioral resistance.

In studies conducted on several pests species, such as coleopteran maize weevils or dipteran parasites, the results on behavioral resistance show that it is neither correlated negatively [22] nor positively [23] with the physiological resistance to insecticides. The development of mechanisms of behavioral
and physiological resistance to pesticides must be considered adaptive events and independent each other [43], although in both cases they are transmitted genetically to the offspring [12]. However, the results indicate that certain species would lack the capacity to develop behavioral resistance, such as the snakefly, *H. laufferi*. This makes this species much more susceptible to pesticides and it would explain its absence in the conventional olive grove. A greater knockdown effect of pesticides on insects without behavioral resistance, such as *H. laufferi*, makes them excellent ecological indicators of organic management.

**Conclusions**
The results of this study indicate that the induction of behavioral resistance through the application of experimental treatments performed on a small scale, represents a very useful tool to qualify the type of management to which a given crop is being subjected.

On the one hand, it allows to determine if the lineages of the species of natural enemies present in a certain culture have developed behavioral resistance. This is what would be expected in those crops in which conventional pesticides have been used or are being used. In the opposite case, the absence of behavioral resistance allows to assure that a certain crop is a good example of organic agriculture. The absence of certain species, such as raphidiptera, in crops subjected to regular use of conventional pesticides, makes them excellent indicators of organic agriculture.

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