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Response of two tephritid species, *Bactrocera oleae* and *Ceratitis capitata*, to different emission levels of pheromone and parapheromone

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Running title: Pheromone response in fruit flies

Abstract.

Attractants and pheromones are commonly used in integrated pest management programs in crop systems. However, pheromone dispensers employed in monitoring traps and lure and kill devices are not usually well studied and attractants are released at uncontrolled rates leading to low treatment efficacies and misleading monitoring estimations. Fruit flies are pests of economic importance and monitoring is essential in order to program insecticidal treatments. Moreover, lure and kill techniques are being increasingly used, but the cost of these techniques depends on the number of required traps and, therefore, on the efficacy of the attractants. *Ceratitis capitata* and *Bactrocera oleae* are the two main fruit flies in Mediterranean countries, and the effect of different doses of trimedlure and spiroacetal on fly attraction has been studied. Results showed that a release rate over 1.28 mg/day of spiroacetal reduces *B. oleae* attraction and emission values over 2.4 mg of trimedlure per day did not increase *C. capitata* catches. Under the environmental conditions of our study, an optimum release rate for pheromone attraction in *B. oleae* was determined. Emission values over this optimum level reduced *B. oleae* attraction. However, when a parapheromone was used with *C. capitata*, a fruit fly of the same family, the optimum emission value was not found and higher quantities of parapheromone attracted the same number of flies. The saturation effect of high concentrations of pheromone and parapheromone is discussed.

KEYWORDS. *Ceratitis capitata; Bactrocera oleae*; pheromone; attractant; monitoring; lure&kill.

1. Introduction

Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) and Olive fruit fly, *Bactrocera oleae* Gmelin are two major pests of Mediterranean agriculture. *B. oleae* is a monophagous fruit fly which appears in several countries where *Olea europaea* L. is grown. It is endemic in the Mediterranean area but in recent decades it has invaded the olive growing areas in California and Mexico. It is also found in South and Central Africa, Pakistan and the Middle East (Daane and Johnson, 2010; Nardi et al., 2005). Females of the olive fruit fly are the only tephritid females known to produce a sex pheromone, with 1,7-dioxaspiro[5.5]undecane (hereafter spiroacetal) as its major component (Baker et al., 1980; Jones et al., 1983; Haniotakis et al., 1994). Male olive flies also produce this compound, attracting other males, but females are not attracted to spiroacetal emitted from either sex (Haniotakis et al., 1994). In order to monitor olive fly populations, two types of traps baited with two kinds of substances are used. McPhail or Olipe traps baited with a mixture of ammonium bicarbonate or ammonium diphosphate are employed for female monitoring. Sticky boards baited with spiroacetal are used for male flight monitoring. In general, males are monitored in the Mediterranean area between May and December when mating activity takes place (Jones et al., 1983).

C. capitata is a polyphagous pest in temperate climates all around the world, with the exception of central and East Asia, with 4 to 10 generations per year depending on the latitude. The *C. capitata* female pheromone to attract males has not been identified. Males are responsible for female attraction (Landolt et al., 1992) and much research aimed at finding new substances released by medfly males to attract females has been conducted (Jacobson et al., 1973; Ohinata et al., 1977; Baker et al, 1985; Jang et al., 1989). A study on the sexual behavior of *C. capitata* described how the male raises the tip of his abdomen and emits a long-distance attractant pheromone before mounting the female (Briceño and

Eberhard, 2002). Research on the composition of male pheromone emissions is still on-going, and recent studies have provided lists which contain the composition of aeration samples of calling males (Gonçalves et al., 2006; Alfaro et al., 2011). In spite of these efforts, full pheromonal attractiveness is not achieved in field tests with blends using major constituents of these pheromone emissions (Light et al., 1999). This would mean that minor components could be having a key effect, and that, therefore, the high complexity of this pheromonal blend makes it very difficult to find an effective mixture.

Trimedlure (TML) was described in 1964 as an attractant for Mediterranean fruit fly (Beroza et al., 1964). TML is a sex-specific attractant widely used in detection and monitoring programmes around the world. Ceralure, an iodinated analog of TML was developed by McGovern et al. (1988) and more recent studies demonstrated that the (-) enantiomer of ceralure B1 was more effective than trimedlure as a male attractant (Jang et al., 2003). Both trimedlure and ceralure are parapheromones, and no disruption effects have been described although they have been extensively used as male attractants.

Aside from mating disruption, attractants and pheromones have been used in "attract and kill", chemosterilant (Navarro-Llopis et al., 2007, 2010) or infective devices, but mass trapping is the most widespread technique (El-Sayed et al., 2009). For both fruit flies, the efficacy of this technique depends on the type of trap and the attractant. Several studies have examined the effect of the design and color of traps on their efficacy for monitoring tephritids (Epsky et al., 1995; Cornelius et al., 1999; Navarro-Llopis et al., 2008), but only a few studies have associated pheromone release rate and insect catches (Landolt and Heath, 1990; Dominguez-Ruiz et al., 2008; Suckling et al., 2008). Many studies have compared catches among several types or loads of dispensers for other insect families (Cork et al., 2000; Franklin and Gregoire, 2001; Kovanci et al., 2006), but only a few

determined the optimal release rate of attractants in field trials (de Groot and DeBarr, 1998; Cross et al., 2006; Vacas et al., 2009).

The optimal pheromone release rate is not well known in most cases. Some pheromone release threshold values to achieve mating disruption depending on the target pest can be found in the literature (Ioratti et al., 2004; de Lame and Gut, 2006; Stelinski et al., 2007). The key to improve control methods based on pheromones as attractants (mass trapping or monitoring) is to know the optimal emission rate, because catches could decrease below and above this optimal value (Jacobson and Beroza, 1964; Roelofs and Carde, 1977; Zhang and Amalin, 2005). However, there are not many conclusive studies on this subject. The aim of our study was to obtain the optimal release rate which maximizes the efficacy of an attractant for the control of fruit flies. For this purpose, four levels of pheromone emission were compared using traps baited with a different number of standard commercial pheromone dispensers. The efficacy of each trap was measured in field trials as the number of fruit fly catches. These four emission levels were correlated with field captures to evaluate the existence of an optimal emission rate for both spiroacetal and TML.

2. Material and methods

2.1 Olive fruit fly

2.1.1. Traps and pheromones.

Olive fruit flies were captured using yellow sticky traps made of a polyethylene vinyl acetate (PVC) yellow plastic sheet measuring 220 x 175 mm, and coated on both sides with non drying glue (provided by Biagro S.L. ,Valencia, Spain).

For the pheromone release study, polyethylene vials (PD) were selected, with a specification of 80 mg (a.i.) for spiroacetal, and were provided by Aragro S.A. (Madrid,

Spain). These dispensers were chosen due to their release pattern, which is constant over time. This allows the study of fly catches over a period of several weeks with minimal variation in release rate. Pheromone dispensers were inserted in the center of the sticky trap.

2.1.2. Field trial.

A field trial was carried out in a 5 ha 10 year old *O. europaea* orchard with trees spaced at 8 by 10 m (125 trees/ha), located in Betera (Valencia, Spain). The orchard was divided into four plots to study the effect of four different emission levels in four blocks, placing four traps in a row inside each block. Therefore, each block contained four traps baited with 1, 2, 3 or 4 spiroacetal dispensers. The separation distance between traps was 32 m within each block and their position was randomized in the first plot. The position of traps in the rest of the plots was designed to avoid having traps with the same number of dispensers in the same position. The separation between plots was at least 40 m. Catches were recorded and traps were rotated within each plot every week from October to December 2009.

2.2 Mediterranean fruit fly

2.2.1. Traps and pheromones.

Mosquisan® traps were used for trapping and were provided by Sansan Prodesing S.L. (Valencia, Spain). This trap was evaluated and described by Navarro-Llopis et al. (2008). Pheromone dispensers Zentinel Ceca® were provided by Ecologia y Protección Agrícola S.L. (Carlet, Spain). The dispensers were mesoporous (MD) cylindrical tablets containing 1.8 g of TML, which were described in Dominguez et al (2008). In that study, the quantification of isomers was included. Each trap contained the corresponding number of TML dispensers and a 500 mg DDVP strip from Agrisense BCS Ltd. (Portypridd, UK).

2.2.2. Field trial.

The field trial was carried out between July and October 2009, when the *C. capitata* population was high enough to obtain a representative number of catches, and citrus fruits began to ripen. Traps were placed in a 6 ha, 20 year old citrus grove (*Citrus reticulata* Blanco, variety Marisol), with trees spaced at 6 by 4 m (420 trees/ha), located in Sagunto, near Valencia on the east coast of Spain. The field trial included four plots to study the effect of different TML emission levels. Plots were arranged following the same experimental design as for the *B. oleae* trial. Traps with 1, 2, 4 or 6 TML dispensers were placed in each plot. Traps were separated by 30 m to avoid both direct interaction between traps and natural spatial population variation. Separation between plots was at least 100 m. *C. capitata* catches were recorded every week, and traps were rotated within each block.

2.3. Release rates

For release rate measurement, 40 dispensers of each type were aged in the same areas, 500 m away from the trial orchards. TML dispensers were aged inside the same Mosquisan® traps and spiroacetal dispensers were attached to sticky traps as in the monitoring traps. Both traps were hung from trees and four aged dispensers were transferred weekly to the laboratory. Residual pheromone was extracted at 0, 5, 7, 10, 14, 21, 28, 35, 42, 49, 55 and 62 days of aging from spiroacetal dispensers and 0, 15, 23, 38, 52, 67 and 82 days for TML dispensers. Three dispensers of spiroacetal and TML for each aging time were extracted. The extraction method used for spiroacetal dispensers was solvent extraction using the Extraction Unit Soxtec® 2043 System (Rose Scientific Ltd. Alberta, Canada). Extraction conditions consisted of 2 hours of extracting followed by 2 hours of washing at 50 ºC with dichloromethane. The TML content from dispensers was obtained by regular solvent extraction. Each dispenser was placed inside a 100 ml tube with a magnet and 50

ml of dichloromethane. The extraction procedure used magnetic agitation at 500 r.p.m. during 2 hours, after which extracts were centrifuged at 3,000 r.p.m for 8 min.

Spiroacetal and TML were quantified by gas chromatography with flame ionization detector (GC/FID), using 1-dodecanol and 3-octanol as internal standards respectively. All injections were made in a Clarus500 gas chromatograph from PerkinElmer Inc. (Wellesley, MA) using a ZB-5 column (30 m × 0.25 mm i.d. × 0.25 μm; Phenomenex Inc., Torrance, CA). The carrier gas was helium at 1.2 ml/min. Retention times of TML and spiroacetal were confirmed with commercial standards provided by Ecologia y Protección Agrícola (Carlet, Spain).

2.4. Statistical analysis

Multiple linear regressions were applied to study the evolution of residual pheromone and parapheromone loads versus time for each type of dispenser. To determine whether the emission was constant during the time under study, the significance of the quadratic effect was checked.

The main aim of this work was to study the correlation of the factor emission level with trap catches. For this purpose, variability of catch data due to other factors was first studied by applying a multifactor analysis of variance (ANOVA) to evaluate the effect of date and block factors on the data. The square-root transformation of the number of catches was used to normalize the data. The part of the variability not explained by these two factors will remain on the residuals of the ANOVA. These residuals were used in a subsequent multiple regression analysis to study the effect of factor emission, with four levels for *B. oleae* (1 PD, 2 PD, 3 PD and 4 PD) and *C. capitata* (1 MD, 2, MD, 4 MD and 6 MD). Then, the existence of a relative maximum was investigated. This methodology had already been applied in Vacas et al (2009). Statistical analyses were performed using the Statgraphics plus 5.1 package.

3. Results

3.1. Release Rates

Figures 1 and 2 show the evolution of the remaining load of spiroacetal and TML, respectively, versus time for the commercial dispensers. The residual spiroacetal and TML loads were fitted by simple linear regression, resulting in R^2 = 0.999 for spiroacetal (F=9381.59; d.f.=1,9; P<0.001) and R^2 = 0.918 for TML (F=40.12; d.f.=1,5; P=0.001). In the case of TML, it was observed that data at day = 0 appeared as outliers, which is explained by the higher emission rate during the first days until the MD dispenser reaches equilibrium with the environment within one or two weeks. This condition did not exist in the spiroacetal PD dispensers, which were a different type of dispenser. The TML contained in the surface of the MD was likely released faster. However, the pheromone is inside the polyethylene tube in the PD, not impregnated on the surface, so spiroacetal must pass through the polyethylene walls to be released. Release rate of TML dispensers was not constant during the first two weeks, thus data for the first 15 days were discarded for TML dispensers. Quadratic effect was not significant for spiroacetal and TML release rates: P = 0.07 (F=3246.25; df=2,4; P>0.001 for spiroacetal) and P = 0.09 (F=43.98; df=2,4; P=0.006 for TML) in the multiple regression analysis . Thus, it was assumed that the residual pheromone load decreased at a constant rate over the studied period. The emission rates for each dispenser were the slopes of their respective linear model. To obtain a better estimation of the coefficients, a multiple linear model was fitted using the type of dispenser as an indicator variable (F=40.12; df=1,5; P=0.001 for TML and F=9381,59; df=1,5; P<0.001 for spiroacetal). The resulting slopes (± standard errors) were 2.360±0.341 mg/day for the TML dispenser and 0.463±0.005 mg/day for the spiroacetal dispenser.

3.2 Field Trial

3.2.1. Trap Catches.

Figures 3 and 4 show the average number of catches obtained in traps baited with spiroacetal and TML commercial dispensers respectively. For spiroacetal dispensers, the olive fly catches went down significantly from the 4th week to the end of the trial (Fig. 3). Therefore, only data from the first three weeks were considered for statistical analysis because such low values do not give sufficiently reliable information to study the effects of block or emission.

Only the catches retrieved from to 3rd to 12th week (Fig. 4) were considered to simplify *C. capitata* statistical analysis because, as mentioned above, TML emission was not constant during the first two weeks of the trial.

3.2.2. Olive fruit fly.

A multifactor analysis of variance (ANOVA) was used to evaluate the effect of date and block factors on olive fly catches. Date factor showed statistical significance ($F=67.71$; $df=2,65$; $P<0.001$): captures of weeks 1st to 3rd were significantly higher than the rest because this period corresponds to the maximum pest population (Fig. 3). The block effect was also statistically significant ($F=4,71$; $df=5,62$; $P = 0.001$): this effect can be explained by the barrier effect of the traps described for other tephritids (Cohen and Yuval, 2000), producing that the average number of catches in the two central blocks was significantly lower than in the two external blocks.

The part of the variability not explained by these two factors will remain on the residuals of the ANOVA. These residuals were used in a subsequent multiple regression analysis to study the effect of factor emission, with four levels for *B. oleae* (1 PD, 2 PD, 3 PD and 4 PD). Then, the existence of a relative maximum was investigated.

Emission factor also showed statistical significance (F=3.79; df=3,64; P=0.014). The means plot and LSD intervals for this factor (Fig. 5) show that the highest release rate, emitted from traps baited with 4 PD, captured significantly less than traps baited with 3 PD. This result suggested that attractant efficacy decreases above a particular emission value. Taking into account that the estimated emission rate of the PD dispensers was 0.46 mg/day, the emission factor was considered as a quantitative variable according to this correspondence: 1 PD = 0.46 mg/day, 2 PD = 0.92 mg/day, 3 PD = 1.38 mg/day and 4 PD = 1.84 mg/day.

The residuals of the previous ANOVA analysis were used to perform a multiple regression analysis to study the linear and quadratic effect of emission, obtaining the equation 1:

$$Residuals = -2.13\,x^2 + 5.48\,x - 2.91$$

The coefficient of the quadratic term in this equation was statistically significant (P = 0.03) in the multiple regression test (F=3.47, df=65,2; P=0.037), which reflects a curvature in the model and confirms the existence of an optimum value of emission that maximizes attractant activity. To obtain this value, equation 1 was derived and equalled to zero, resulting in an optimum of 1.28 mg/day. Figure 5 shows the curvature that best fits the 4 mean values of captures according to the emission rate.

3.2.3. Mediterranean fruit fly.

As described in olive fruit fly, a multifactor analysis of variance (ANOVA) was used to evaluate the effect of date and block factors on Mediterranean fruit fly catches. Date factor showed statistical significance (F=21.92 ; df=8,152 ; P<0.001), probably due to natural population dynamics and to the natural dispersion of *C. capitata*. Captures during the first two weeks were significantly higher than the rest because this period corresponds to the maximum pest population (Fig. 4). The effect of block factor was also statistically significant (F=8.88 ; df=3,152 ; P<0.001) and, as in the *B. oleae* study, the average

number of fruit fly catches in the two central blocks was significantly lower than in the two external blocks.

Emission was not statistically significant (F=0.91; df=140,3; P = 0.43). Figure 6 shows that the trap baited with 1 MD captured more flies than the others, but these differences were not significant. This result suggests the lack of a maximum attraction value, so the attractant power does not decrease above a particular release value. Taking into account that the estimated emission rate of the MD dispensers was 2.36 mg/day, the emission factor was considered as a quantitative variable according to this correspondence: 1 MD = 2.36 mg/day, 2 MD = 4.72 mg/day, 4 MD = 9.44 mg/day and 6 MD = 14.16 mg/day. Figure 6 was obtained using the same methodology as for *B. oleae*. In this figure, no significant reduction of *C. capitata* catches could be observed by increasing the number of TML dispensers (F = 1.26; df = 140,3; P = 0.29). In addition, lineal and quadratic effects are not significant (P = 0.24 and P = 0.35 respectively) in a polynomial regression (F=1.34; df=2,141; P=0.26) .

In the case of medfly the attractant power did not decrease above a particular emission value. The minimum tested concentration was 2.36 mg/day because it was established as an optimum concentration for *C. capitata* attraction (Dominguez-Ruiz et al., 2008). Although this release rate was multiplied by 6, significant differences among emission levels were not detected.

4. Discussion

Different correlations of field trap catches with pheromone release rates were obtained in this work. *C. capitata* response reached a maximum with a release rate near 2 mg/day of its parapheromone TML and increasing emission levels did not increase or reduce fly

catches. By contrast, *B. oleae* attraction to traps baited with spiroacetal increased up to a limit, with decreasing responses to the highest emission level.

Haniotakis and Pittara (1994) found in laboratory tests that *B. oleae* male responses changed according to different pheromone loads. In this study, the maximum male response was obtained with an intermediate dose of filter paper dispensers releasing 0.59 mg/h of 1,7-dioxaspiro [5.5] undecane, which suggested the existence of an optimal release level. In addition, it was observed that response to the optimum pheromone concentration decreased significantly after 1 min of continuous exposure to the pheromone which means a saturation effect. However, actual release rates and the responses of flies in the field were not measured in this work. There are some studies showing a maximum attraction at a particular pheromone release rate, in Lepidoptera (Vacas et al., 2009), Coleoptera (Obengofori, 1990; Franklin and Gregoire, 2001; Cross et al., 2006) or other Diptera (Michaelakis et al., 2007). The importance of the determination of optimum pheromone release rates has already been shown in research by Landolt and Heath (1988, 1990), testing behavioural responses of the tephritid female Papaya fruit fly, *Toxotrypana curvicaudata* Gerstaecker. In laboratory bioassays, the authors found increasing responses to increasing pheromone release rates up to a determined emission value. Later, this result was confirmed in field trials with an optimum rate of 960 ng/h (Landolt and Heath, 1990). The present study supports the existence of these optimum release values in some tephritid species. Specifically, an optimum pheromone release value of 1.28 mg/day was determined for the attraction of *B. oleae* in the West-Mediterranean conditions in the two months before harvesting. This value may vary under different environmental conditions, so more replications in different locations should be carried out in order to study variations in this optimum.

Currently, monitoring of olive fruit fly males is carried out in Spain with a pheromone dispenser in the top or in the middle of yellow sticky boards. These dispensers are usually polyethylene vials or rubber septa with loads of pheromone ranging from 1 to 80 mg, having lifespans from 40 to 120 days. These different loads indicate substantial variability in release rates and the resulting estimates of fruit fly populations. This would mean that olive fly populations could be over or underestimated depending on the dispenser used. Thus, the type of pheromone dispenser must be taken into account to calculate treatment thresholds. In addition, if manufacturers change dispenser formulations, the calculation of treatment thresholds would result in vain. The optimum pheromone release rate, maximizing *B. oleae* captures, was established by correlating pheromone release rates and field fly catches. This information improves trapping efficiency, so that monitoring results can be optimized. Moreover, this optimum value will be useful in studies correlating fruit damage with trap catches without dependence on the type of dispenser.

The use of optimum release values is very important to test the actual potential or efficacy of mass trapping programs. Up to now, many mass trapping field trials have been carried out using commercial monitoring dispensers, without information about the attractant release rate (Haniotakis et al., 1991; Broumas et al., 2002). On the other hand, it must be mentioned that release rates are influenced by the type of trap used in the tests (Jones at al., 1983). Therefore, the optimum emission value obtained in this work is only useful for sticky boards and should be recalculated for McPhail, delta or Jackson traps, as aeration of the attractant might be different.

Another factor affecting fly catches is the physiological state of the insects. The field trials in our study were conducted over the main part of the season for these pests; that is two months before citrus harvesting in the *C. capitata* trial and one month before olive harvesting in the *B. oleae* trial. During these periods of the year, fruit fly populations are

usually monitored to determine whether population thresholds are exceeded and if insecticidal treatments should be applied. Moreover, the maturation of eggs and oviposition of *B. oleae* females occurs during this period when maximum male catches are detected (Torres-Vila et al, 2006).

As stated in the introduction, *B. oleae* females are the only tephritid known with a well defined pheromone. In this way, a mating disruption technique could be applied, in principle. This study supports this idea because a decrease of fly catches was detected with high pheromone concentrations. This means that olive fruit flies cannot find the source of odor when pheromone concentration is very high and this is the basis of mating disruption. However, mating disruption studies carried out against *B.oleae* had inconclusive results in Spain and Greece (Montiel et al, 1982; Montiel and Jones, 2002).

Regarding *C. capitata*, the disruption effect was not observed when different emission levels were tested for the parapheromone TML. The highest catches were obtained with dispensers releasing TML around 2 mg/day. Fly catches did not however significantly decrease with the TML release rate increasing more than 6 times. This lack of saturation in response to higher pheromone concentrations could explain why TML do not produce flight disruption in *C. capitata.*

The effect of parapheromones on target insects is very similar to the effect of pheromones, but the specificity of parapheromones is lower. In many cases, substances such as cuelure or methyl eugenol attract many organisms of the same genus, unlike pheromones which are species-specific. Moreover, parapheromones cause a stimulus in the sensilia of the insect which could be as powerful as pheromones, but in most cases this stimulus stops after a few seconds. In the case of pheromones, this effect is very strong in the sensilia and the stimulus could remain much longer giving a high EAD signal. This means that pheromone specificity is very high and the joint place of the pheromone to the receptor

can be saturated, whereas parapheromone receptors do not become saturated (Quero et al, 2004). This could explain the existence of an optimum dose for pheromones, as higher doses saturate the receptors, whereas the emission rate with parapheromones could be increased without saturating the receptors.

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FIGURE FOOTNOTES

Fig. 1 Release dynamics of spiroacetal from commercial polyethylene dispensers.

Fig. 2 Release dynamics of trimedlure from commercial mesoporous dispensers.

Fig. 3 Average Olive fruit fly catches per trap per week obtained in yellow PVC sticky boards baited with commercial spiroacetal dispensers.

Fig. 4 Average Mediterranean fruit fly catches per trap per week obtained in Moskisan® traps baited with mesoporous TML dispensers.

Fig. 5 Captures of *B. oleae* and 95% LSD intervals corresponding to factor emission for spiroacetal release rates. Curve represents the quadratic model that best fits the mean values of captures according to emission rates. Significance of quadratic term was P=0.03.

Fig. 6 Captures of *C. capitata* and 95% LSD intervals corresponding to factor emission for trimedlure release rates. Interval overlapping indicates the lack of a maximum attraction value. Polynomial regression shows that lineal and quadratic effect are not significant.