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Determination of the baseline susceptibility of European populations of *Cydia pomonella* (Lepidoptera: Tortricidae) to chlorantraniliprole and the role of cytochrome P450 monooxygenases.

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Short title: Baseline susceptibility of *Cydia pomonella* to chlorantraniliprole

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Abstract

The codling moth, *Cydia pomonella*, (L.) (Lepidoptera: Tortricidae) is the key pest on pome fruit and walnut orchards worldwide. Its resistance to available insecticides has been widely reported. Chlorantraniliprole is an anthranilic diamide that was introduced in European countries in 2008-2009 and acts by activating the insect's ryanodine receptors.

The aims of this study were to determine the baseline susceptibility of European populations of *C. pomonella* to chlorantraniliprole, to establish the discriminant concentrations (DC) to check the possible development of resistance, and to know the role of cytochrome P450 monooxygenases (P450) in the possible susceptibility decrease of field populations to the insecticide.

Ten field populations from Spain along with others were used to calculate the baseline response of larvae to chlorantraniliprole incorporated into the diet. A pooled probit line was calculated and three DC were established: 0.3 mg a.i./kg (close to the LC_{50}), 1.0 mg a.i./kg (close to the LC_{90}), and 10 mg a.i./kg diets (three-fold the LC_{99}). The DC were used to test the susceptibility of 27 field populations from France, Germany, Hungary, Italy and Spain. The corrected mortality observed in all cases ranged within the expected interval, even with Spanish populations that showed between 12.1% and 100.0% of individuals with high P450 activity. However, the mortality caused by the $DC_{0.3}$ decreased as the mean P450 activity increased. Field populations resistant to other insecticides were susceptible to chlorantraniliprole. The determined baseline codling moth susceptibility is a valuable reference for tracking possible future alterations in the efficacy of the insecticide.

KEY WORDS: Codling moth, chlorantraniliprole, baseline, resistance monitoring

Introduction

The codling moth (CM), *Cydia pomonella*, (L.) (Lepidoptera: Tortricidae) is the key pest on pome fruit and walnut orchards in almost all the areas where these crops are cultivated. At present, its control is mostly achieved by a combination of pesticides and mating disruption (Witzgall et al., 2008; Ioriatti & Lucchi 2016). The existence of CM insecticide-resistant populations is a widespread problem in many pome fruit production areas in the world, and it concerns 10 out of the 11 insecticide modes of action available to control this pest, depending on the country (IRAC Codling Moth WG, 2013), which are: 1A – carbamates, 1B – organophosphates, 3A – pyrethroids, 4A – neonicotinoids, 5 – spinosyns, 6 – avermectins, 7B – phenoxy-ethyl carbamates, 15 – benzoylureas, 18 – diacylhydrazines and 22A oxadiazines (Sauphanor et al., 1998, 2000; Dunley and Welter, 2000; Reuveny & Cohen, 2004; Fuentes-Contreras et al., 2007; Ioriatti et al., 2007; Stará & Kocourek, 2007; Knight, 2010; Rodríguez et al., 2010, 2011, 2012; Voudouris et al., 2011; Grigg-McGuffin et al., 2015; Iscy and Ay, 2017). Codling moth resistance to insecticides is mainly metabolic, due to three enzymatic complexes (cytochrome P450 monooxygenases (P450), glutathione transferases (GST) and esterases (EST)) (www.irac-online.org), but mutations in the insecticide target site protein have also been detected (the acetylcholinesterase (*AChE*) mutation, only reported in the fruit growing area of Lleida (Spain) (Cassanelli et al., 2006), and the knockdown resistance (*kdr*) mutation (Brun-Barale et al., (2005)). For example, when compared to non-chemically treated orchards, an increase of the frequency of resistant codling moth individuals has been detected in many chemically treated orchards in the tree fruit area of Lleida, and enhanced P450 activity was the main enzymatic mechanism involved (Rodríguez et al., 2010, 2011; Bosch et al., 2016). However, enhanced GST and EST activity was also detected (Rodríguez et al., 2011), as the presence of *AChE* and *kdr* mutations (Bosch et

al., 2014). Metabolic cross-resistance is a big concern in any management resistance strategy, which may occur either at the level of interaction with the various chemical families or at a geographical level (Dunley and Welter, 2000; Reyes et al., 2007; Voudouris et al., 2011; IRAC Codling Moth WG, 2013). New pesticides with new modes of action and with an environmentally safe toxicological profile are then necessary to control resistant CM populations.

Chlorantraniliprole (Rynaxypyr®) and cyantraniliprole (Cyazypyr®), developed by DuPont (USA), are anthranilic diamides whose mode of action has been classified in the new insecticide group 28 (ryanodine receptor modulator) within the IRAC (Insecticide Resistance Action Committee) mode of action classification scheme (Nauen, 2006). By activating the insect ryanodine receptors (RyRs), they stimulate the release and depletion of intracellular calcium stores from the sarcoplasmic reticulum of muscle cells, causing impaired muscle regulation, paralysis and, ultimately, death (Cordova et al., 2006). Chlorantraniliprole acts primarily by ingestion and by contact on the larvae of chewing pests. Newly hatched larvae from treated eggs die when eating the chorion at emergence. It has extremely broad-spectrum effectiveness within the insect order Lepidoptera and some Coleoptera, Diptera and Isoptera, acting in a broad range of crops and showing very low mammalian toxicity and high selectivity to non-target arthropods (DuPont™, 2008; Lahm et al, 2009). Chlorantraniliprole is active on the codling moth, and it has been registered in European countries since 2008. Due to the potential of *C. pomonella* to develop resistance to insecticides, it is necessary to establish a baseline susceptibility database before or early after the introduction of a new insecticide to the market (Roditakis et al., 2013). The database has to include data from a wide geographical area, to know the natural variability of the response to the chemical in

93 field populations, and it has to consider the state of the insecticide resistance in every
94 area.
95 The aims of this study were to determine the baseline susceptibility of European codling
96 moth populations to chlorantraniliprole, to establish the discriminant concentrations
97 (DC), to check the possible development of resistance in the field, and to understand the
98 possible role of P450 in the lack of susceptibility of field populations to this insecticide.

99

100 ## Material and methods

101 ### *Experimental protocol outline*

102 In our study, the concentration – response to chlorantraniliprole lines (mortality vs.
103 concentration) by ingestion were first determined for eight Spanish field populations
104 collected from 2007 to 2009. These data along with others obtained from populations in
105 Italy, France, Netherlands and Belgium and tested since 2005 in Italy were used by
106 DuPont® to calculate the Discriminant Concentrations (DC). Values close to the LC_{50},
107 LC_{90}, and to three times the LC_{99} were chosen as the DC. These concentrations were then
108 applied to 27 European populations collected from 2008 to 2015 to test their
109 susceptibility. Two more concentration – response lines were later determined for two
110 more Spanish populations collected in 2010. Their DC results were within the range of
111 the ones calculated previously and all the probit line data were pooled to calculate a
112 common probit line. Additionally, the probit line of chlorantraniliprole was also
113 calculated for a Spanish susceptible strain (S-Spain) whose response to several
114 insecticides is well known. Finally, the activity of P450 was measured for 12 field
115 populations and for the laboratory susceptible strain.

116

Insects

The list of the CM field populations used in the bioassays is shown in Table 1. The 10 Spanish populations used to adjust probit lines were collected from 2007 to 2010 in the Ebro Valley pome fruit production area (Catalonia and Aragon, NE of Spain) when the product was not registered. The 27 populations tested with the DC were collected from 2008 to 2015 in the pome fruit growing areas of France, Germany, Hungary, Italy, and Spain. They were collected before or after the registration of chlorantraniliprole, or from orchards that had not been sprayed with it.

Except in one case (Jun 3-63 (09), that was collected from an abandoned orchard), the Spanish and most of the rest of the European field populations originated from commercial IPM orchards. The pest management strategy with chlorantraniliprole was the same in all countries, once the product was registered: only two applications per season on one single generation, preferable first generation. Most of the CM field populations were collected as diapausing larvae using corrugated cardboards installed in the field from July–August until October, but in some cases injured apples were collected or corrugated cardboards were installed in the field to obtain non diapausing larvae or both. When it was needed, the populations were reared until their second generation (F2) to have enough progeny to carry out the bioassays (Table 1).

The susceptible CM strain, S-Spain, was collected from an abandoned apple orchard in Lleida in 1992, and it has been reared since then using a semi-artificial dehydrated apple diet at the joint IRTA and UdL laboratory (Lleida, Spain). Its response to many insecticides, its P450, GST and EST enzymatic activity and the presence of the *AChE* mutation are well known (Rodríguez et al., 2010; Rodríguez et al., 2011; Bosch et al., 2014).

141 The P450 activity in CM adults was determined for 12 field populations from the Ebro
142 Valley (Spain). Codling moth adults from the first flight were captured from seven
143 orchards in pheromone traps in 2009 and 2010 (Table 1). As for the other five populations
144 collected in 2010 and 2011, the P450 activity was measured on adults emerging from the
145 collected diapausing larvae.

146 *Insecticide and bioassays experimental procedure*

147 Chlorantraniliprole was used as DPX-E2Y45 20SC (Coragen® 20SC, DuPont de
148 Nemours France SAS, Nambsheim, France) and a Stonefly Premix® (Stonefly Industries
149 LTD) lyophilized diet was used as feeding substrate in the bioassays, following the IRAC
150 susceptibility test method 017 (www.irac-online.org) To complement the diet, CD
151 International BA-128 multiwell plastic trays (each well of 15.9 mm diameter and 15.9
152 mm deep) and lids were used. Codling moth neonate larvae (less than 24-h old) were
153 exposed to chlorantraniliprole in diet incorporated assays.
154 To calculate the baseline probit lines 20 grams of diet were mixed with 60 g of the
155 insecticide solution at given concentrations ranging from 0.01 to 1.0 mg a.i./kg of diet at
156 approximately 3x series dilution. Given the results, a second series was performed to get
157 a better fitting of the probit line. Distilled water was used as a solvent. Each well was
158 filled with 0.5 g of treated diet, and the diet was pressed to be evenly distributed across
159 the bottom. One neonate larvae per well was placed on the treated diet. At least three
160 replications of 16 larvae were tested per each concentration. The trays were incubated at
161 22 ± 1 ºC, 16:8 h (L:D) and 45% humidity. Larval mortality was assessed after four days.
162 Larvae were considered dead when they did not move after a light touch with a brush or
163 when they were moribund. Moribund larvae were those visibly affected and significantly
164 different from normal ones; when they were probed and flipped on their back, the larva
165 could not flip back right-side up, or, when it was able to do it, it did so with uncoordinated

and slow movements. Only data in which control mortality was <20% were analyzed. Missing larvae were subtracted from the initial number.

Once the DC were calculated, to test the susceptibility of 27 European field populations the same procedure was followed.

P450 enzymatic activity

The adult P450 activity was determined in the susceptible strain S-Spain and in 12 field populations (Table 1) with an *in vivo* protocol (Rodríguez et al. 2012) using 7-ethoxy-coumarin-O-deethylation (ECOD) in a black microplate of 96 wells. The dissected abdomens of the adults were placed individually in a well containing 100 µL of phosphate buffer (50 mM, pH 7.2) and 7-ethoxycoumarin (0.4 mM). After 4 h of incubation at 30 °C, the reaction was stopped by adding 100 µL of glycine buffer (pH 10.4, 10-4 M) with ethanol (v/v). Before the incubation, a minimum of 10% of the wells of each microplate were used as controls and received the glycine buffer to stop the reaction. The ECOD activity was measured by fluorescence with a 380 nm excitation filter and 465 nm emission filters and was expressed as pg of 7-ethoxycoumarin (7OH).insect^{-1}.min^{-1}.

Data analysis

To calculate the baseline a probit analysis using the program POLO Plus (LeOra Software, 1987) was performed, and the LC_{50}, the LC_{90} and their 95% fiducial limits were calculated. Two LC_{50} were considered significantly different when their fiducial limits did not overlap (Robertson *et al.* 2007). The resistant ratio (RR) relative to the most susceptible field population (RR-F_{50}) and the resistance ratio relative to the susceptible laboratory strain (RR-L_{50}) were calculated for the LC_{50}. Values close to the LC_{50}, to the LC_{90}, and to three times the LC_{99} were chosen as the DC. The highest DC was expected to kill approximately 100% of larvae of the susceptible population, but a smaller percentage when applied to a resistant population (ffrench-Constant and Roush, 1990).

191 To evaluate the susceptibility of the European field populations the corrected mortality
192 produced by the DC was calculated using Abbott's formula (Abbott, 1925), being the
193 correction factor the mortality produced by the control treatment (water). The RR of each
194 population for every DC was calculated by dividing the mortality of the most susceptible
195 field population by the mortality produced in every field population strain (RR-$F_{0.3}$, RR-
196 $F_{1.0}$ and RR-$F_{10.0}$). Mean ± SEM of the corrected mortality produced by the DCs and the
197 coefficient of variation (CV) across all the field populations to evaluate the dispersion of
198 the data were calculated.

199 The frequency of P450-resistant individuals in every CM field population analysed was
200 compared to the susceptible S-Spain using a Pearson chi-square ($\chi 2$) test. Moths were
201 classified as resistant if their P450 enzyme activity exceeded the highest activity value
202 corresponding to 90% of S-Spain individuals (Reyes et al., 2007). Regression lines
203 between the mean P450 activity (pg 7OH insect^{-1} min^{-1}) and the LC_{50}, and between the
204 P450 activity and the mortality produced by the $DC_{0.3}$ for each population were
205 calculated. Only the orchards with more than 20 adults analyzed were taken into account
206 for the regression lines.

207

208 **Results**

209 The results of the probit analysis and the RR are shown in Table 2. The LC_{50} values for
210 the CM field populations ranged from 0.161 to 0.446 mg a.i./kg diet. Some significant
211 differences were found among the field populations, namely Torref 15-3 (SP) (08), Bal
212 2-480 (SP) (08) and SAS (SP) (09) were significantly less susceptible than five field
213 populations, and Riud (SP) (10) was significantly more susceptible than four field
214 populations. One population, Torreg 11-160 (SP) (08), showed no significant differences

with any of the other field populations. Jun 3-63 (SP) (09), from an abandoned orchard, showed no significant differences with six of the ten tested field populations. The RR-F_{50}, calculated comparing each LC_{50} value with the LC_{50} value of the most susceptible field population (Riud (SP) 10, 0.161 mg a.i./kg diet), ranged from 1.1 to 2.8.. The laboratory susceptible strain, S-Spain, was significantly more susceptible to chlorantraniliprole than any field population and it was not included in the calculation of the pooled LCs values. The RR-L_{50}, calculated comparing each LC_{50} value with the LC_{50} value of the laboratory strain (S-Spain, 0.086 mg a.i./kg diet) ranged from 1.9 to 5.2. SAS (SP) (09) had a high slope (4.860 ± 0.557) suggesting a more homogeneous response than the other populations and a narrow concentration range between the LC_{50} and LC_{90}. The same happened with Riud (SP) (10), S-Spain and Tam (SP) (10) that had parallel slopes to SAS (SP) (09) ($\chi 2 = 3.34$, d.f. = 3, $P < 0.343$). Torreg 11-160 (SP) (08) had the lowest slope (1.371 ± 0.168) compared to the next smallest one, Torref 15-3 (SP) (08) ($\chi 2 = 4.80$, d.f. = 1, $P < 0.028$). Due to its low slope Torreg 11-160 (SP) (08) had a very wide interval of concentrations between LC_{90} and LC_{50} and thus, had high intrapopulation variability and it did not present significant differences with any other field population. The LC_{90} values for the CM field populations ranged from 0.339 to 2.788 mg a.i./kg diet.

The LC_{50} value of the pooled data was 0.250 mg a.i./ kg of diet, the LC_{90} was 0.888 mg a.i./ kg of diet and the LC_{99} was 3.323 mg a.i./ kg of diet. These results are according to the DC selected to test the susceptibility of CM field populations to chlorantraniliprole: 0.3 and 1.0 mg a.i./kg diets, approximately the LC_{50} and the LC_{90}, respectively, and 10 mg a.i./kg diet which corresponded to three-fold the LC_{99} value of the pooled data probit line results.

Table 3 shows the corrected mortality and the resistance ratio relative to the most susceptible field population (RR-Fs) obtained with each DC for the European CM field

240 populations. All the DC applied produced a 100% of mortality on the susceptible
241 population S-Spain, as expected according to its probit line results (Table 2). Feeding the
242 European field populations larvae with concentrations of 0.3, 1.0 and 10.0 mg a.i./kg diet
243 produced mean corrected mortalities of 70.82%, 96.44% and 99.85%, respectively. The
244 CV were low for 0.3 mg a.i./kg diet (31.17), and very low for 1.0 (7.21) and 10.0 mg
245 a.i./kg diets (0.52). The RR-$F_{0.3}$ ranged between 1.1 and 4.8, and between 1.0 and 1.5 for
246 the other two DC.

247 The P450 enzymatic activity of CM adults and the relative frequency of resistant
248 individuals (those whose P450 activity was higher than 23.92 pg 7OH $adult^{-1}$ min^{-1}) are
249 shown in Table 4. The mean P450 activity ranged from 10.52 to 74.61 pg 7OH $insect^{-1}$
250 min^{-1}, and the frequency of resistant individuals in the field populations ranged from
251 12.1% to 100.0% (populations Jun 3-63 (SP) (09) and Balaguer 2-480 (SP) (08) for both
252 variables, respectively). The variance of the frequency of resistant insects is strongly
253 explained by the P450 mean activity of the field populations (R^2 = 95.32), which implies
254 that there are few individuals with a very high resistance level that strongly influence the
255 mean P450 value (Figure 1). Although data were restricted to four field populations, the
256 regression line between the frequency of resistant insects and the LC_{50} values showed a
257 high coefficient of determination (R^2 = 89.77, Figure 2). When the regression line was
258 adjusted between the P450 mean activity and the mortality produced by the $DC_{0.3}$, the
259 coefficient of determination was lower than in the other cases, but it explained the 69.82%
260 of the variance (Figure 3). The regression lines obtained with the higher DCs (1.0 mg
261 a.i./kg of diet and 10.0 mg a.i./kg of diet) hadn't good adjustments due to the high
262 mortalities obtained in all the field populations treated.

263

264

Discussion

To determine the DC, populations collected before the registration of chlorantraniliprole were used. The first European countries to get the product registered on pome fruits were Romania in 2008 and Italy in 2009, and in Spain the product was registered in 2011. The CM Spanish field populations showed a high and relatively uniform susceptibility to chlorantraniliprole. This is likely because of the RR relative to the most susceptible population (RR-F_{50}) was lower than 2.8, even though significant differences on the LC_{50}s were observed among populations (Mota-Sanchez et al., 2002). All the field populations, even the abandoned orchard Jun 3-63 (SP) (09), were significantly less susceptible to chlorantraniliprole than the laboratory strain, S-Spain. Ffrench-Constant and Roush (1990) stated that susceptible strains held for long periods in the laboratory may bear little resemblance to susceptible strains currently found in the field. Thus, it would be more useful to determine the appropriate DC on the basis of field strains before wide commercial introduction of the specific pesticide. Therefore, to establish a useful baseline to know the natural response variability to the insecticide in the field populations, the laboratory susceptible strain was not included in the pooled data to determine the DCs. Nevertheless, once the product is applied in the field, susceptibility of insect populations might reduce over time due to potential resistance evolution (ffrench-Constant and Roush, 1990). In this case, susceptible strains can be helpful as an invariable mortality reference point, which are commonly used (Zheng et al., 2011; Lai et al., 2011; da Silva, 2012; Caballero et al., 2013).

The LC_{50} values obtained varied between 0.086 mg a.i./kg of diet (S-Spain) and 0.446 mg a.i./kg of diet (Torref 15-3 (SP) (08)), and are equivalent to 0.110 mg a.i./L and 0.59 mg a.i./L, respectively. The same insecticide diet incorporation bioassay obtained an LC_{50} value range in different laboratory susceptible strains of different species of

290 Lepidoptera: 0.014 mg a.i./L in *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae)
291 (Lai et al., 2011) and 0.28 mg a.i./L in *Choristoneura rosaceana* (Harris) (Lepidoptera:
292 Tortricidae) (Sial and Brunner, 2012). Different species respond in a different way to
293 the same insecticide, independently of the body mass differences. For example, CM is
294 heavier than *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae) but
295 females of CM were 115 times more susceptible to thiacloprid than females of *L.*
296 *botrana* were (Navarro et al., 2017). In the laboratory, where the larval feeding behavior
297 is conditioned, these different responses are mainly due to that different species may
298 have different metabolic procedures or detoxification methods (Rodríguez et al., 2012).
299 The highest resistance ratio relative to the laboratory strain of S-Spain (RR-L_{50}) was 5.2
300 (Table 2) which is a low value compared to the highest value observed for Chinese *S.*
301 *exigua* field populations (17.1, Lai et al., 2011) in which chlorantraniliprole was either
302 briefly introduced or, in some cases, never used against. As found our study, narrow
303 variations in the LC_{50} values were also found in some other Lepidoptera species: *Tuta*
304 *absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (Campos et al., 2015), *Plutella*
305 *xylostella* (Linnaeus) (Lepidoptera: Plutellidae) (Wang et al., 2010) or *Cnaphalocrocis*
306 *medinalis* (Güenée) (Lepidoptera: Pyralidae) (Zheng et al., 2011).
307 The mortality caused by the $DC_{0.3}$ (concentration close to the LC_{50} of the pooled field
308 population) on European field populations also showed a low variability among
309 populations, with a mean mortality of 70.82 %, and a CV equal to 31.17 (Table 3). The
310 RR-$F_{0.3}$ obtained were similar to the RR-$F_{50,}$ demonstrating its reliability in comparing
311 populations. The $DC_{1.0}$ produced a mean mortality of 96.44%, and all the mortalities were
312 higher than 85.3% except in the French population, Isle SLS (FR) (13), where it was
313 67.7%. The highest DC tested, 10 mg a.i./kg of diet, caused 100.0% mortality in all the
314 field populations except in Isle SLS (FR) (13) and Malpartit (SP) (09), suggesting the

315 presence of a small proportion of resistant individuals in these two populations, but
316 generally supporting the lack of resistance to chlorantraniliprole in the field. Malpartit
317 (SP) (09) was collected before the introduction of the product in the marked but the
318 populations had a very high mean P450 activity (Table 4), what can play a role in the
319 detoxification of the active ingredient, as is discussed later. Eleven European field
320 populations were collected after registration of the product, from 2012 onwards, and these
321 populations proved as susceptible as the previously tested Spanish populations. Some of
322 them were collected over more than a year, as in: Le Thor (two years), Isle SLS (three
323 years) and Noves P (five consecutive years). The mortality obtained with Isle SLS with
324 the DC of 0.3 mg a.i./kg of diet decreased over the years from 89.1% in 2009 to 38.1%
325 in 2013. This population was from a location with very high pest pressure and where trials
326 with Rynaxypyr were done over several years, so, the loss of efficacy of the product may
327 be due to the presence of resistant individuals in the population. Nevertheless, the field
328 population, Noves, showed a similar level of mortality for all the years, ranging between
329 69.6% to 93.3%, except in 2012, when the efficacy of the $DC_{0.3}$ decrease to 20.8%
330 (obtaining a $RR_{0.3}$ of 4.8). In these field populations, the $DC_{1.0}$ and $DC_{10.0}$ reached 100.0%
331 of mortality suggesting an error in the assay or an unexplained variation in the mortality
332 obtained, something that can occur in unexposed field populations (Sawicki, 1987) and
333 demonstrating the utility of using more than one DC.

334 Widespread resistance of CM field populations from the Ebro Valley area (NE of Spain)
335 has been demonstrated, mainly to azynphos-methyl and other OP, lambda-cyhalothrin
336 and methoxyfenozide, among other active ingredients (Rodríguez et al., 2010;
337 Rodríguez et al., 2011). Insecticide bioassays (Bosch et al., 2017) were done with some
338 of the Spanish field populations tested with chlorantraniliprole. Compared with the
339 susceptible population S-Spain, PuigverdB (11) was resistant to the pyretroid lambda-

cyhalothrin (RR = 15.4), and PuigverdC (11) was resistant to lambda-cyhalothrin (RR = 872.0), methoxyfenozide (RR = 14.6) and thiacloprid (RR = 11.2), besides being tolerant (2 < RR < 10) to other active ingredients such as chlorpyrifos-ethyl and spinetoram. Tam (10) was susceptible to all the tested active ingredients, and SAS (11) and Le Thor (12) were tolerant to different active ingredients but with RRs always lower than 4.4. None of these populations were resistant to chlorantraniliprole, with SAS (11) the only one that had a RR slightly higher than 2 (RR = 2.2). Despite being multi-resistant populations, PuigverdB (11) and PuigverdC (11) responded to the DCs of chlorantraniliprole with high mortalities, at the same or a higher level than the rest of the tested populations (Table 3). These results showed the absence of cross-resistance among chlorantraniliprole and lambda-cyhalothrin, methoxyfenozide and thiacloprid in these field populations suggesting that the resistant mechanisms involved do not affect the proper activity of the product. This lack of cross-resistance was also found in *P. xylostella* field and laboratory selected populations (Wang et al., 2010), *Spodoptera litura* (Fabricius) (Lepidoptera: Tortricidae) (Sang et al., 2016) and with cyantraniliprole, another anthranilic diamide, in selected resistant populations of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Grávalos et al., 2015). Therefore, a high correlation between the two anthranilic diamides was found in *T. absoluta* (Campos et al., 2015) and in *S. litura* (Sang et al., 2016); and with flubendiamide, a phthalic diamide, in *P. xylostella* although this active ingredient had never been used (Wang et al., 2013). Chlorantraniliprole is the only diamide currently registered for pest control in pome fruits, but formulated products of cyantraniliprole are under development to control a cross-spectrum of chewing and sucking pests from the insect orders Hemiptera, Lepidoptera and Coleoptera (Selvy et al., 2013). They have obtained promising results in the control of aphid pests with no evidence of cross-

365 resistance with other aphid insecticides (Foster et al., 2012). In the case of using both
366 active ingredients in the same crop to control different pests, an intensive check to
367 detect an increase in the resistance levels would be necessary due to the possible cross-
368 resistance between IRAC Group 28 products, together with an accurate resistance
369 management strategy combining its use.

370 With reference to enzymatic detoxification mechanisms, synergism assays in *S. litura*
371 (Su et al., 2012), *S. exigua* (Lai et al., 2011) and *P. xylostella* (Wang et al., 2010)
372 demonstrated that P450, EST and GST were not the main mechanisms involved in
373 chlorantraniliprole resistance and neither were they involved in the cyantraniliprole
374 resistance of *S. litura* in China (Sang et al., 2016). However, in *T. absoluta*, Campos et
375 al. (2015) found a moderate correlation between the cytochrome-P450-monooxygenases
376 activity (P450) and a susceptibility to chlorantraniliprole and cyantraniliprole. Sial et al.
377 (2011) found that EST could be involved in the detoxification of chlorantraniliprole in a
378 resistant selected laboratory population of *C. rosaceana*; and Cao et al. (2010) found
379 that there was an increase of EST and GST activity in chlorantraniliprole treated insects
380 of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). The main enzymatic
381 mechanism for insecticide detoxification of the Spanish CM field populations is P450
382 (Rodríguez et al., 2010; Rodríguez et al., 2011), and in the studied populations, the
383 frequency of P450 resistant insects present in the field explained the 69.72% of the
384 mortality obtained with the lower chlorantraniliprole DC (0.3 mg a.i./kg of diet). The
385 coefficient of determination was higher when the LC_{50} was used instead of the mortality
386 of the $DC_{0.3}$ ($R^2 = 89.77$); however, we considered that five points (the laboratory and
387 four field populations) to adjust a regression line are too few to draw any conclusion.
388 Despite these results, due to the high efficacy of the product in the tested field

389 populations, P450 seems not to be the main mechanism implied in the detoxification of
390 the product although it may have a certain role in it.

391 *C. pomonella* is a key fruit pest that has extensively demonstrated its ability to develop
392 resistance to most of the registered insecticides. Chlorantraniliprole, a new reduced risk
393 insecticide that can control a wide range of lepidopteran pests, has proved its high
394 efficacy in European field populations by obtaining low RR and variability when the LC
395 were calculated and DC tested. The efficacy of the product in this assay not only has
396 shown the natural variability in the response concentration-mortality in a broad
397 geographical area, but also the lack of cross-resistance of the product with other
398 commonly used insecticides in Spanish field populations, such as lambda-cyhalothrin,
399 methoxyfenozide or thiacloprid. Nevertheless, it seems there is a relationship between
400 the frequency of resistant individuals due to high P450 enzymatic activity and the
401 mortality produced by the approximate LC_{50} used as a DC. As many insecticides can
402 induce an P450 enzymatic activity increase, the use of a strict resistance management
403 strategy would be necessary to maintain the efficacy of the product for a long time
404 (http://www.irac-online.org). In fact, this strategy has been considered from the
405 beginning by DuPont which recommend a restricted number of applications per season
406 on the same generation, within spray programs that include other effective insecticides
407 with different modes of action. The CM baseline susceptibility data established provides
408 a valuable reference for tracking possible future alterations in the efficacy of the
409 product.
410

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Table 1. Population name, origin, year of collection, state of the insects collected (DP = diapausing) and generation tested of the field codling moth populations treated with chlorantraniliprole to obtain either the probit line (Probit) or the mortality produced by the discriminant concentrations (DC). The P450 activity was calculated for the populations reported in column: P450 activity.

Population	Country / County	Collection year	Insect collection state	Tested laboratory generation	Bioassay done	P450 activity
S-Spain	Spain / Catalunya				Probit	Yes
Bal 2-371 (SP) (07)	Spain / Catalunya	2007	DP larvae	F1	Probit	
Jun 14-16 (SP) (08)	Spain / Catalunya	2008	Non DP & DP larvae *	F2	Probit	Yes
Cal-4 (SP) (08)	Spain / Aragón	2008	Non DP & DP larvae	F2	Probit	
Torref 15-3 (SP) (08)	Spain / Catalunya	2008	Non DP & DP larvae *	F2	Probit	Yes
Torreg 11-160 (SP) (08)	Spain / Catalunya	2008	Non DP & DP larvae	F2	Probit	
Bal 2-480 (SP) (08)	Spain / Catalunya	2008	Non DP & DP larvae *	F2	Probit	Yes
SAS (SP) (09)	Spain / Catalunya	2009	DP larvae	F2	Probit	
Jun 3-63 (SP) (09)	Spain / Catalunya	2009	Non DP & DP larvae *	F1	Probit	Yes
Tamarite (SP) (10)	Spain / Aragón	2010	Non DP & DP larvae	F1	Probit	Yes
Riud (SP) (10)	Spain / Catalunya	2010	DP larvae	F1	Probit	
Torreg 11-166 (SP) (09)	Spain / Catalunya	2008	DP larvae *	F2	DC	Yes
La Almunia (SP) (09)	Spain /Aragón	2009	DP larvae	F1	DC	
La AlmuniaG (SP)(09)	Spain /Aragón	2009	DP larvae	F2	DC	
Tossal (SP) (09)	Spain / Catalunya	2009	Non DP & DP larvae *	F2	DC	Yes
Malpartit (SP) (09)	Spain / Catalunya	2009	Non DP & DP larvae *	F1	DC	Yes
Vauvert (FR) (09)	France /Languedoc-Roussillon	2009	DP larvae	F2	DC	
Isle SLS (FR) (09)	France / Vaucluse	2009	DP larvae	F2	DC	
Nedel Market (HU)(09)	Hungary / Bács-Kiskun	2009	DP larvae	F2	DC	

*Adults caught in pheromone traps

Table 1 cont. Population name, origin, year of collection, state of the insects collected (DP = diapausing) and generation tested of the field codling moth populations treated with chlorantraniliprole to obtain either the probit line (Probit) or the mortality produced by the discriminant concentrations (DC). The P450 activity was calculated for the populations reported in column P450 activity.

Population	Country / County	Collection year	Insect collection state	Tested laboratory generation	Bioassay done	P450 activity
Aseleben (DE) (09)	Germany / Sachsen Anhalt	2009	DP larvae	F1	DC	
Isle SlS (FR) (10)	France / Vaucluse	2010	DP larvae	F1	DC	
Noves P (FR) (10)	France / Provence-A-C.A.	2010	DP larvae	F1	DC	
Albalate (SP) (11)	Spain /Aragón	2011	Non DP & DP larvae	F2	DC	Yes
PuigvertC (SP) (11)	Spain / Catalunya	2011	DP larvae	F1	DC	Yes
PuigvertB (SP) (11)	Spain / Catalunya	2011	DP larvae	F1	DC	Yes
SAS (SP) (11)	Spain / Catalunya	2011	DP larvae	F1	DC	Yes
Noves P (FR) (11)	France / Provence-A-C.A.	2011	DP larvae	F1	DC	
Noves P (FR) (12)	France / Provence-A-C.A.	2012	DP larvae	F1	DC	
Le Thor (FR) (12)	France / Provence-A-C.A.	2012	DP larvae	F1	DC	
Isle SLS (FR) (13)	France / Vaucluse	2013	DP larvae	F2	DC	
Noves P (FR) (13)	France / Provence-A-C.A.	2013	DP larvae	F2	DC	
Noves P (FR) (14)	France / Provence-A-C.A.	2014	DP larvae	F1	DC	
Lumpiaque (SP) (15)	Spain /Aragón	2015	DP larvae	F1	DC	
Salillas (SP) (15)	Spain /Aragón	2015	DP larvae	F1	DC	
Le Thor (FR) (15)	France / Provence-A-C.A.	2015	DP larvae	F1	DC	
Meckenheim (DE) (15)	Germany	2015	DP larvae	F1	DC	
Orsingen (DE) (15)	Germany	2015	DP larvae	F1	DC	
Ravenna (IT) (15)	Italy	2015	DP larvae	F1	DC	

*Adults caught in pheromone traps

Table 2. Baseline susceptibility of *Cydia pomonella* Spanish field collected populations to chlorantraniliprole. n = number of individuals tested. RR-F_{50} = resistance ratio calculated by dividing the LC_{50} of the strain tested by the LC_{50} of the most susceptible field population (Riud (SP) (10)). RR-L_{50} = resistance ratio calculated by dividing the LC_{50} of the strain tested by the LC_{50} of the susceptible laboratory population (S-Spain). Values of the LC are mg a.i./kg diet. LC_{50} followed by the same letter are not significantly different (LC_{50} are considered significantly different when their CI do not overlap).

Population (year)	n	Probit analyses parameters								RR-F_{50}	RR-L_{50}
		Intercept	Slope ± SE	LC_{50}	CI 95%	LC_{90}	CI 95%	χ2 (dF)	P*		
S-Spain	577	3.555	3.345 ± 0.252	0.087	0.079-0.095	0.209	0.182-0.249	0.36 (4)	> 0.05	-	-
Bal 2-371 (SP) (07)	275	1.606	2.220 ± 0.396	0.189ab	0.124-0.254	0.714	0.488-1.502	0.71 (4)	> 0.05	1.2	2.2
Jun 14-16 (SP) (08)	572	1.102	2.183 ± 0.260	0.313bc	0.220-0.426	1.209	0.786-2.861	5.09 (6)	> 0.05	1.9	3.6
Cal-4 (SP) (08)	612	1.974	2.800 ± 0.269	0.197ab	0.160-0.243	0.566	0.427-0.867	8.03 (6)	> 0.05	1.2	2.3
Torref 15-3 (SP) (08)	400	0.684	1.954 ± 0.260	0.446c	0.309-0.665	2.021	1.173-6.365	5.63 (5)	> 0.05	2.8	5.2
Torreg 11-160 (SP) (08)	262	0.671	1.371 ± 0.168	0.324abc	0.182-0.592	2.788	1.302-11.396	9.31 (3)	< 0.05	2.0	3.8
Bal 2-480 (SP) (08)	436	0.809	2.079 ± 0.248	0.408c	0.312-0.536	1.687	1.114-3.531	5.70 (5)	< 0.05	2.5	4.7
SAS (SP) (09)	380	2.235	4.860 ± 0.557	0.347c	0.251-0.431	0.636	0.503-1.051	37.47 (4)	< 0.05	2.2	4.0
Jun 3-63 (SP) (09)	283	2.041	2.722 ± 0.317	0.178ab	0.137-0.230	0.526	0.383-0.849	4.22 (3)	> 0.05	1.1	2.1
Tam (SP) (10)	684	2.624	3.748 ± 0.486	0.199ab	0.162-0.248	0.438	0.332-0.749	4.99 (4)	> 0.05	1.2	2.2
Riud (SP) (10)	554	3.139	3.951 ± 0.337	0.161a	0.136-0.185	0.339	0.258-0.431	11.42 (5)	< 0.05	1.0	1.9
Pooled	4458	1.401	2.324 ± 0.087	0.250	0.225-0.376	0.888	0.763-1.068	186.73 (50)	< 0.05	1.6	2.9

*The p-value >0.05 indicates that the observed bioassay data are not significantly different from the expected data for the probit model.

Table 3. Susceptibility, expressed as corrected mortality (%), of *Cydia pomonella* European field collected populations to discriminant concentrations of chlorantraniliprole (0.3, 1.0 and 10.0 mg a.i./kg diet). RR-F = resistance ratio for every DC was calculated by dividing the mortality of the most susceptible field population (Meckenheim (DE) (15)) by the mortality produced in every field population strain. Mean ± SEM of the corrected mortality produced by the DCs and coefficient of variability (CV) across all the field populations were calculated. Numbers in brackets are the number of individuals tested.

Population	Control mortality (%)	Discriminant concentration (mg a.i./kg diet)					
		0.3	RR-$F_{0.3}$	1.0	RR-$F_{1.0}$	10.0	RR-$F_{10.0}$
S-Spain		100.0 (44)		100.0 (47)		100.0 (48)	
Torreg 11-166 (SP) (08)	4.2 (48)	44.3 (45)	2.3	97.8 (48)	1.0	100.0 (69)	1.0
La Almunia (SP) (09)	0.0 (50)	78.6 (47)	1.3	95.7 (47)	1.0	100.0 (46)	1.0
La Almunia G (SP) (09)	2.5 (40)	36.9 (53)	2.7	97.1 (52)	1.0	100.0 (68)	1.0
Tossal (SP) (09)	6.7 (45)	35.4 (48)	2.8	97.7 (44)	1.0	100.0 (48)	1.0
Malpartit (SP) (09)	2.4 (42)	51.0 (46)	2.0	93.6 (16)	1.1	97.8 (46)	1.0
Vauvert (FR) (09)	0.0 (48)	54.2 (48)	1.8	85.3 (44)	1.2	100.0 (49)	1.0
Isle SLS (FR) (09)	0.0 (48)	89.1 (46)	1.1	100.0 (47)	1.0	100.0 (47)	1.0
Nedel Market (HU) (09)	6.1 (50)	93.3 (48)	1.1	96.5 (51)	1.0	-	-
Aseleben (DE) (09)	1.6 (64)	90.9 (59)	1.1	100.0 (60)	1.0	100.0 (64)	1.0
Isle SlS (FR) (10)	2.1 (48)	48.9 (40)	2.0	98.0 (50)	1.0	100.0 (48)	1.0
Noves P (FR) (10)	2.0 (49)	69.6 (47)	1.4	100.0 (48)	1.0	100.0 (45)	1.0
Albalate (SP) (11)	0.0 (48)	69.4 (48)	1.4	100.0 (48)	1.0	100.0 (48)	1.0
PuigvertC (SP) (11)	8.3 (48)	81.4 (47)	1.2	93.0 (47)	1.1	100.0 (48)	1.0
PuigvertB (SP) (11)	14.6 (48)	95.0 (47)	1.1	100.0 (47)	1.0	100.0 (48)	1.0

Table 3 cont. Susceptibility, expressed as corrected mortality (%), of *Cydia pomonella* European field collected populations to discriminant concentrations of chlorantraniliprole (0.3, 1.0 and 10.0 mg a.i./kg diet). RR-F = resistance ratio for every DC was calculated by dividing the mortality of the most susceptible field population (Meckenheim (DE) (15)) by the mortality produced in every field population strain. Mean ± SEM of the corrected mortality produced by the DCs and coefficient of variability (CV) across all the field populations were calculated. Numbers in brackets are the number of individuals tested.

Population	Control mortality (%)	Discriminant concentration (mg a.i./kg diet)					
		0.3	RR-$F_{0.3}$	1.0	RR-$F_{1.0}$	10.0	RR-$F_{10.0}$
SAS (SP) (11)	0.0 (48)	82.5 (48)	1.2	100.0 (48)	1.0	100.0 (48)	1.0
Noves P (FR) (11)	4.7 (64)	79.9 (47)	1.3	100.0 (63)	1.0	100.0 (46)	1.0
Noves P (FR) (12)	0.0 (62)	20.8 (48)	4.8	100.0 (48)	1.0	100.0 (48)	1.0
Le Thor (FR) (12)	4.7 (64)	75.4 (47)	1.3	100.0 (63)	1.0	100.0 (46)	1.0
Isle SLS (FR) (13)	2.1 (48)	38.1 (61)	2.6	67.7 (79)	1.5	98,9 (95)	1.0
Noves P (FR) (13)	0.0 (48)	82.2 (45)	1.2	100.0 (47)	1.0	100.0 (48)	1.0
Noves P (FR) (14)	0.0 (48)	93.3 (45)	1.1	100.0 (33)	1.0		
Lumpiaque (SP) (15)	4.3 (82)	77.8 (63)	1.3	87.2 (122)	1.1	100.0 (45)	1.0
Salillas (SP) (15)	0.0 (27)	72.3 (47)	1.4	100.0 (55)	1.0	100.0 (55)	1.0
Le Thor (FR) (15)	0.0 (64)	64.8 (88)	1.5	94.3 (138)	1.1		
Meckenheim (DE)(15)	8.3 (48)	100.0 (53)	1.0	100.0 (45)	1.0	100.0 (48)	1.0
Orsingen (DE) (15)	0.0 (16)	93.2 (44)	1.1	100.0 (45)	1.0		
Ravenna (IT) (15)	4.2 (96)	93.7 (95)	1.1	100.0 (94)	1.0		
Mean ± SEM		70.82 ± 4.25		96.44 ± 1.34		99.85 ± 0.11	
CV		31.17		7.21		0.52	

Table 4. Enzymatic P450 activity, expressed as frequency of resistant insects (%) and mean activity ± SEM (pg 7OH·insect^{-1}·min^{-1}), of *Cydia pomonella* field adults. The value of the P450 threshold obtained using the S-Spain population was 23.92 pg 7OH·insect^{-1}·min^{-1}. The frequency of resistant individuals was compared using a Pearson $\chi 2$ test (df = 1; *p = 0.05; **p = 0.01; ***p = 0.001).

Population (year)	n	Resistance frequency (%)	Mean P450 activity ± SEM	LC_{50}	$DC_{0.3}$ mortality (%)
S-Spain	223	10.0	9.64 ± 0.74	0.086	100.0
Jun 14-16 (08)	47	66.0***	34.20 ± 3.18	0.313	51.8
Torref 15-3 (08)	125	76.0***	54.47 ± 2.95	0.446	34.2
Bal 2-480 (08)	8	100.0***	74.61 ± 9.57	0.408	49.3
Jun 3-63 (09)	107	12.1n.s.	10.52 ± 0.98	0.178	83.0
Torreg 11-166 (09)	48	45.8***	31.52 ± 4.50	-	44.3
Tossal (09)	41	85.4***	62.05 ± 6.24	-	35.4
Malpartit (09)	82	91.0***	62.34 ± 3.87	-	51.0
Tam (10)	29	31.0**	20.60 ± 2.73	0.192	75.6
SAS (11)	29	20.7n.s.	16.60 ± 2.68	-	82.5
Albalate (11)	23	27.6***	20.20 ± 3.23	-	69.4
PuigverdC (11)	39	53.9***	30.00 ± 3.34	-	81.4
PuigverdB (11)	29	48.3***	28.31 ± 3.24	-	81.4

Figure 1. The frequency of resistant codling moth adults as explained by the mean enzymatic P450 activity in 11 Spanish field populations and the laboratory strain S-Spain.

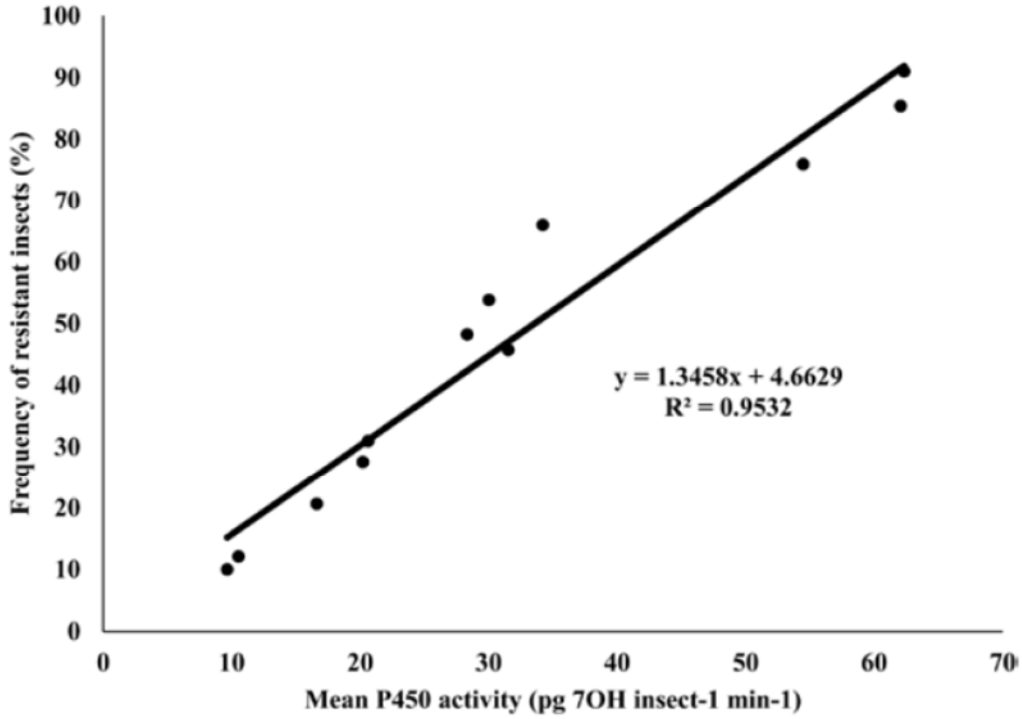

Figure 2. The LC_{50} of chlorantraniliprole for four Spanish field populations and the laboratory strain S-Spain as explained by the frequency of resistant codling moth adults.

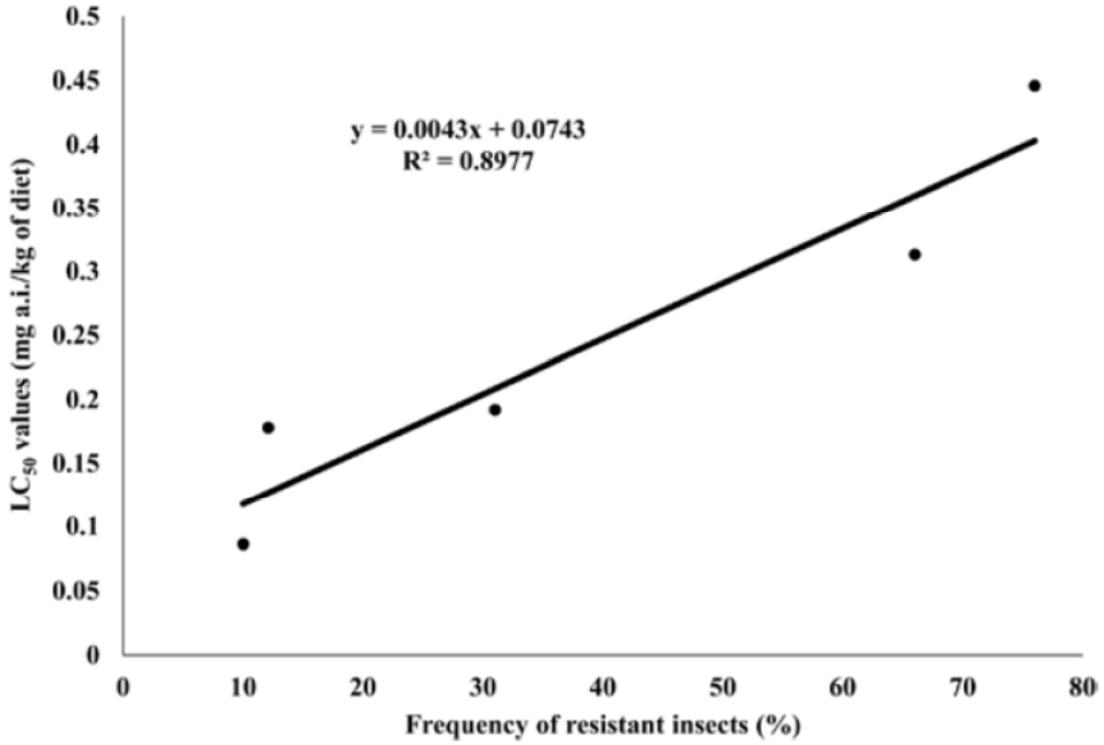

Figure 3. The mortality caused by the chlorantraniliprole $DC_{0.3}$ on larvae of 11 Spanish field populations and the laboratory strain S-Spain as explained by the mean enzymatic P450 activity in adults.