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Title :

Metabolic mechanisms and acetylcholinesterase sensitivity involved in tolerance to chlorpyrifos-ethyl in the earwig Forficula auricularia

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Highlights:

- Chlorpyrifos-ethyl exposure killed fewer earwigs from conventional orchard
- High carboxylesterases / glutathione-S-transferases found in pre-exposed earwigs
- Acetylcholinesterase was less sensitive in earwigs from conventional orchard
- Chlorpyrifos-induced inhibition of acetylcholinesterase was higher in males

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Abstract

Apple orchards are highly treated crops, in which organophosphorus (OP) are among the most heavily sprayed insecticides. These pesticides are toxic to non-target arthropods and their repeated use increases the risk of resistance. We studied mechanisms involved in tolerance and resistance to OP insecticides in the earwig *Forficula auricularia,* an effective generalist predator in pomefruit orchards. Adult earwigs were sampled in three apple orchards managed under contrasting strategies: conventional, Integrated Pest Management, and organic. The threshold activities of enzyme families involved in pesticides tolerance: Glutathione-S-transferases (GSTs) and Carboxylesterases (CbEs) were measured in earwig extracts. Acetylcholinesterase (AChE) was monitored as a toxicological endpoint. Variations in these activities were assessed prior to and after exposure to chlorpyrifos-ethyl at the normal application rate. We observed that the mortality of earwigs exposed to chlorpyrifos-ethyl depended on the management strategy of orchards. Significantly lower mortality was seen in individuals sampled from conventional orchard. The basal activities of CbEs and GSTs of collected organisms were higher in conventional orchard. After *in vivo* exposure, AChE activity appeared to be inhibited in surviving males with no difference between orchards. However an *in vitro* inhibition trial with chlorpyrifos-oxon showed that AChE from earwigs collected in organic and IPM orchards were more sensitive than from conventional ones. These observations support the hypothesis of a molecular target modification in AChE and highlight the possible role of CbEs in effective protection of AChE. Our findings suggest that the earwigs with a high historic level of insecticide exposure could acquire resistance to chlorpyrifos-ethyl.

Keywords: *earwig*; biocontrol agent; tolerance; organophosphorus insecticide

52 # 1. Introduction

53 The widespread use of pesticides in agriculture to control crop pests is having adverse side
54 effects on the environment (Köhler and Triebskorn, 2013). These side effects have been classified
55 into two categories: ecotoxicity (Carson, 1962) and pest resistance to pesticides (R4P, 2016). Rachel
56 Carson was the first scientist to describe ecotoxicity in 1962 in her book the silent spring (Carson,
57 1962). Fifty-seven years later, we are still observing the deleterious effects of pesticides on non-
58 target and beneficial organisms.

59 Apple orchards are highly productive crops. In France, approximately 54,000 ha of apple
60 orchards are sprayed with pesticides with, on average, more than 30 pesticide applications per year
61 in Provence (Sauphanor et al., 2009; Mazzia et al., 2015). Among these treatments,
62 organophosphorus (OP), neonicotinoid and synthetic pyrethroid compounds are the most widely
63 used insecticides.

64 Considered as beneficial insects in apple orchards, earwigs (*Forficula auricularia* (Linnaeus)
65 have been extensively studied due to their key role in the regulation of pests such as aphids, leaf
66 rollers and psyllids (Dib et al., 2010; Dib et al. 2011). Their ease of capture and identification make
67 them an interesting potential bioindicator of orchard management intensity. It has been shown that
68 pesticides, as well as other practices such as tillage and hedge quality, are likely to have an impact on
69 their energy reserves and morphological traits (Suchail et al., 2018), abundance and diversity
70 (Malagnoux et al., 2015a), (Sharley et al., 2008; Moerkens et al., 2012). Several studies have also
71 shown that insecticides directly affect their biochemical (Malagnoux et al., 2014), or behavioral
72 biomarkers such as predatory activity (Sauphanor et al., 1993; Malagnoux et al., 2015b). The impact
73 of pesticides depends on the earwig species: for example, *F. auricularia* is less sensitive to
74 insecticides than *F. pubescens*. Moreover, for both species, a sex-specific response was observed
75 after insecticide exposure (Malagnoux et al., 2015a).

76 OP insecticides, and especially chlorpyriphos-ethyl, are commonly used in agriculture, and
77 they are highly and acutely toxic to wildlife (Köhler and Triebskorn, 2013). These neurotoxic
78 compounds target acetylcholinesterase (AChE) by permanently binding in its active site (Fairbrother,
79 1991; Nunes, 2011). The decrease in AChE activity prevents the hydrolysis of the neurotransmitter
80 acetylcholine (ACh), causing cascade effects such as continuous post-synaptic neuron stimulation and
81 severe neurological disruption. For these reasons, AChE inhibition is the basis of the most popular
82 biomarker of OP insecticide exposure in many vertebrates and invertebrates (Nunes, 2011). After
83 exposure, AChE activity is only restored by de novo enzyme synthesis (Rault et al., 2008).

84 In addition to AChE inhibition and direct impacts on biochemical and behavioral mechanisms
85 in beneficial arthropods, insecticide exposure can increase resistance (Schmidt-Jeffris and Beers,

86 2018) and induce tolerance on organisms (Poupardin et al., 2008). Tolerant populations can undergo
87 rapid phenotypic changes. Such acclimation named phenotypic plasticity is defined by the ability of
88 one genotype to produce different phenotypes in response to time or spatial environmental
89 variability (Kelly et al., 2012). It has been shown that plasticity can induce insecticide tolerance in
90 different organisms such as amphibians (Hua et al., 2013, 2015) and aphid pests (Simon and Peccoud,
91 2018). On another hand, resistance to insecticides can occur via various mechanisms, but
92 modifications to both the detoxification pathways and molecular target of the insecticide are two of
93 the most common ways (R4P, 2016). Two major stages of metabolic transformation of insecticides
94 underlie biotransformation-based acclimation or resistance (Li et al., 2007): (i) a phase of
95 functionalization which results in oxidation by cytochrome P450 multiple function oxidase (CYP_{450})
96 (Puinean et al., 2010). This phase involves a second step, ester hydrolysis or bioscavenging (especially
97 for OP insecticides) by carboxylesterases (CbEs) which can enhance the functionalization stage
98 (Jokanović et al., 1996) or decrease the available OP concentration (Wheelock et al., 2005); and (ii) a
99 second stage named conjugation using glutathione conjugation through the Glutathione-S-
100 transferases (GSTs), the most important enzyme group involved (Kostaropoulos et al., 2001; Enayati
101 et al., 2005). Through these different stages, degradation products are generally less toxic than
102 parent molecules and more easily excretable, but numerous examples also showed an increase in
103 toxicity (Casida, 2016). This was particularly the case for organophosphorus insecticides (OPs), which
104 are activated by CYP_{450} (Costa, 2006).
105 According to Aldridge classification (Aldridge, 1953), CbEs (EC 3.1.1.1.) belong to the B-
106 esterases which are inhibited by OPs while A-esterases are able to hydrolyze OP compounds. CbEs
107 play an important role in the metabolism of many agrochemicals and pharmaceuticals products. In
108 particular, they hydrolyze pyrethroids and they bind stoichiometrically to carbamates and
109 organophosphates (Wheelock et al., 2005). These serine hydrolases are often more sensitive to
110 inhibition by OPs than AChE (Wheelock et al., 2008) so this chemical interaction is considered an
111 efficient mechanism of OP detoxification by which fewer OP molecules may reach the active AChE
112 site. CbEs consist of multiple isozymes with variable levels and activities. Their expression and activity
113 are tissue and organism-dependent, and they can be found in digestive tissues, muscle or brains
114 (Wheelock et al., 2008). It is well known that the variability in CbEs levels as well as relative iso-
115 enzyme abundance contribute to the selective toxicity of ester-containing insecticides in both
116 mammals and insects, and previous results have pointed out their role in the tolerance of some
117 insect pests to OP pesticides (Farnsworth et al., 2010; Reyes et al., 2011).
118 GSTs enzymes (EC 2.5.1.18.) are involved in the detoxification of various endogenous and
119 xenobiotic compounds, such as drugs, insecticides, organic pollutants, secondary metabolites and
120 other toxins (Hayes et al., 2005). They increase the efficiency of conjugation of the cysteine

121 sulphydryl group of glutathione (GSH) with xenobiotics. An electrophilic site of insecticides reacts
122 with this sulphydryl group of GSH leading the formation of GSH conjugates. These conjugates are
123 more readily excreted than the parent insecticide (Sheehan et al., 2001; Hayes et al., 2005). In
124 general, GSTs can be classified into at least four subgroups according to their subcellular location and
125 function: cytosolic, mitochondrial, microsomal and bacterial antibiotic resistance proteins (Sheehan
126 et al., 2001). A majority of insect GSTs are cytosolic enzymes and have been grouped into two main
127 classes (Lumjuan et al., 2007). It is now well established that insect GSTs are associated with
128 resistance to organophosphate, pyrethroids and organochlorine insecticides (Hemingway and
129 Ranson, 2000; Li et al., 2007; Lumjuan et al., 2011). Moreover, increases in GSTs activity and
130 expression levels in pyrethroid resistant mosquitoes have also been reported (Li et al., 2007).

131 Resistance can also be due to target specific mutations. Point mutations in the AChE gene
132 resulting from intensive use of OPs have been identified in many insect species and most are
133 localized in the active site which decreases AChE inhibition (Mutero et al., 1994; Chen et al., 2001;
134 Weill et al., 2003; Anazawa et al., 2003; Li and Han, 2004; Cassanelli et al., 2006). However, the
135 detailed impact of each mutation on the protein structure remains unclear. Point mutations provide
136 specific resistance and depending on the insecticide tested, they may induce resistance or not
137 (Fournier, 2005).

138 The aim of this study was to assess the effects of three orchard management strategies,
139 organic, integrated pest management (IPM) and conventional practices, on *F. auricularia* metabolic
140 pathways and the sensitivity of the AChE target. Both CbEs, and GSTs were investigated as metabolic
141 pathways involved in resistance and/or tolerance. They were studied on earwig populations either
142 before or after exposure to chlorpyrifos-ethyl.

143 2. Materials and methods

144 2.1. Chemicals

145 The 1-naphthyl acetate (1-NA), 1-naphthol, acetylthiocholine iodide (AcSCh), 5,5´-dithiobis-2-
146 nitrobenzoic acid (DTNB), Fast Garnet, reduced glutathione (GSH), 1-Chloro-2,4-dinitrobenzene
147 (CDNB), and a protease inhibitor cocktail were purchased from Sigma–Aldrich (Saint Quentin-
148 Fallavier, France). The pure OP molecule chlorpyrifos-oxon (O,O- diethyl O-3,5,6-trichloro-2-pyridyl
149 phosphate) was purchased from Cluzeau Info Lab (France). For in vivo tests, we used a commercial
150 liquid formulation of the OP insecticide chlorpyrifos-ethyl (Pyrinex ME®, active ingredient 250g a.i. L-
151 1, Philagro).

152 ## 2.2. Earwigs sampling and bio-tests

153 French orchards can be classified into three management practices depending on the type
154 and frequency of pesticides used: 1) organic orchard where all chemical pesticides are replaced with
155 natural products; 2) integrated pest management (IPM), where chemical pesticides are used
156 sparingly according to damage thresholds and finally, 3) conventional orchards (conv), where
157 chemical pesticides are routinely used within the limits of the French law. Within these different
158 strategies we chose 3 commercial orchards (1 orchard per practice), with known contrasted
159 treatments for the last ten years especially concerning the use of OP insecticides (Table 1). All
160 orchards are located in Noves, near Avignon (south-eastern France) within a 10 km radius. Even if
161 very mobile on plants, the dispersal distance of earwigs is very low, less than 30 m per month
162 (Moerkens et al., 2010). This means that we studied 3 distinct populations strongly under the
163 influence of local pesticide use. Earwig populations collected in organic orchards are considered as
164 naïve population regarding OP exposure since they never were exposed to OP treatments, while
165 those from conventional orchard were pre-exposed for years to OP since such treatments were
166 applied more than twice a year every year in the last 20 years except in the last 2 years. Earwigs from
167 IPM are considered as less exposed to OP because after a period of about 1-OP treatment per year,
168 no OP was applied in the IPM orchard since 2013.

169 Male and female adult *F. auricularia* earwigs were collected in July 2017, using cardboard
170 traps placed around the trunk of the apple trees in orchards under the three different management
171 strategies described above: organic, integrated pest management and conventional. Since *F.*
172 *auricularia* species is univoltine, adults were of roughly the same age and care was taken to sample
173 earwigs of similar weight. All bio-test experiments were conducted with Pyrinex® an insecticide
174 authorized in the European Union and commonly used in French non-organic apple orchards until
175 2017. The insecticide solution was diluted with distilled water to adjust the pesticide concentration
176 to the Normal Application Rate (NAR) allowed in French apple orchards (50 $g.hL^{-1}$ active ingredients,
177 chlorpyrifos-ethyl). Petri dishes (8cm diameter) were used for exposure by adapting the method
178 described by Sauphanor et al. (1992), and Malagnoux et al. (2015b). A volume of 115 µL of pesticide
179 solution was applied with a pre-moistened paintbrush on the Petri dish surface to mimic commercial
180 sprayers. The insecticide was left to evaporate for 30 min under an extractor hood to dry the dish
181 before earwig exposure. Control earwigs were exposed to water in the same conditions. For each
182 treatment (control, and chlorpyrifos-ethyl) 50 males and 50 females were placed separately in
183 groups of 5 individuals per Petri dish containing a shelter (small piece of corrugated cardboard) and a
184 piece of food (corn diet) to prevent cannibalism. The Petri dishes were incubated overnight for a 12h
185 exposure time in a climate-controlled chamber at 20°C and subjected to variations in natural light.

186 After this incubation time, the earwig status (alive or dead) was noted. Alive earwigs were frozen at -
187 80°C for biochemical measurements. Earwigs were noted alive when they were able to walk and had
188 moving legs or antenna when physically stimulated with a paintbrush.

189 2.3. Sample preparation

190 The head, thorax and abdomen of the earwigs were dissected and used separately to
191 measure enzyme activities. Abdomens were used for GSTs and CBEs measurements while AChE was
192 measured in the head, which is where this enzyme is produced. Individual tissue samples were
193 homogenized on ice using a homogenizer (Ultra Turrax IKA T18 basic at 14 000 rpm) in 10% (w/v) 50
194 mM Hepes buffer pH 7 for the abdomen or in 10% (w/v) low-salt buffer containing 10 mM Tris– HCl
195 (pH 7.3), 10 mM NaCl supplemented with a cocktail of protease inhibitors for the head (aprotinin,
196 leupeptin and pepstatin = 5 µg mL^{-1}, antipain = 1 µg mL^{-1} , trypsin inhibitor = 1 mg mL^{-1}). Tissue
197 homogenates were centrifuged at 3000 g for 10 min at 4°C and the crude extracts (supernatants),
198 were kept. Abdomen homogenates were immediately used for CBEs and GSTs measurements while
199 head extracts were stored at -20°C with 10% glycerol as an enzyme stabilizing agent until analysis.

200 2.4. Enzyme assays

201 All enzyme activities were measured spectrophotometrically in triplicate using a microplate
202 reader (Synergy HT, Bio-Tek).
203 CBE activity was quantified by measuring the degradation of 1-naphthyl acetate (1-NA) to 1-
204 naphtol (Thompson, 1999). The reaction medium (195 µL final volume) containing 1 µL of diluted
205 (1/20) abdomen extract, 50 mM Hepes buffer (pH 7), 0.03 mM 1-NA (final concentration) was
206 stopped after a 20 min incubation at 25°C by adding 55 µL 2.5% SDS containing 0.04% Fast Garnet
207 GBC. The solutions were left to stand for 20 min at room temperature in the dark, and the
208 absorbance of the 1-naphtol-Fast Garnet complex was read at 590 nm. Enzyme activity was
209 calculated from a standard curve (A590 = f([1-naphtol]) containing pure 1-naphtol as standard, in the
210 same conditions.
211 GST activity was measured at 340 nm for 3 min (Habig et al., 1974) in a reaction mixture (200
212 µL final volume) containing 10 µL of diluted (1/10) abdomen extract, Hepes buffer 50 mM (pH 7),
213 0.75 mM CDNB prepared in ethanol, and 2.5 mM GSH final concentrations.
214 The AChE activity was monitored at 412 nm at 25°C following the method of Ellman et al.
215 (1961) and adapted by Rault et al. (2008), using a millimolar extinction coefficient (6,800 M^{-1} cm^{-1})
216 calculated according to a dithiotreitol-DTNB external calibration curve. The reaction mixture (200 µL
217 final volume) contained 2 µL of head extract, 0.375 mM DTNB, 1.5 mM AcSCh in 0.1 M sodium
218 phosphate buffer (pH=7).

219 Blanks (reaction mixture free of sample) were checked for non-enzymatic hydrolysis of the
220 substrates. The final activities were expressed as units per milligram of total protein ($mU.mg^{-1}$ protein
221 for AChE and $U.mg^{-1}$ protein for CBEs and GST). One unit of enzyme activity was defined as one
222 micromole of formed product per minute under the experimental conditions described above. Total
223 protein content was determined using the Bradford method (Bradford, 1976), with bovine serum
224 albumin as a standard.

225 2.5. In vitro acetylcholinesterase inhibition kinetics

226 *In vitro* inhibition kinetics were conducted to test for a direct interaction between
227 chlorpyrifos-ethyl-oxon and AChE activity. Tissue homogenates from unexposed earwigs (8 µl) were
228 incubated individually with serial concentrations of chlorpyrifos-ethyl-oxon (10^{-12} to 10^{-3} M final
229 concentration) for 30 min at 25ºC. The inhibition assay was terminated by adding the substrate
230 (AcSCh), and the residual AChE activity was measured as described above.

231 2.6. Statistics

232 2.6.1. In vitro inhibition

233 The concentration of inhibitor that inhibited 50% of enzyme activity (IC_{50}) under our specific
234 experimental conditions (pH, temperature, time, protein concentration) was calculated using the
235 logistic curve from the library of non-linear regressions of the XLSTAT software:

236 $y=E_0 \cdot e^{(-b \times [I])}+E_{res}$

237 where E_0 is the total activity, E_{res} the residual activity, $b=k_i \times t$ (k_i inhibition kinetic constant, t
238 the time in min) and $IC_{50}=\ln(2)b^{-1}$.

239 2.6.2. Mortality and activities tests

240 The significance of all results was tested using permutation tests due to non-respect of
241 normality and homoscedasticity (Software; R version 3.4.3 and RStudio version 1.1.423). We used the
242 package 'lmPerm' (Wheeler and Torchiano, 2016) and the function aovp combined with the function
243 pairwise.perm.t.test from RVAideMemoir package. "Fdr" adjust of p-value were used due to risk of
244 multiple comparison.

245 2.6.3. Specificity of mortality data

246 The first aovp test showed that no significant differences were observed for mortality
247 depending on earwig sex (p-value 0.6667). Thus in further analyses and especially for mortality,
248 males and females were grouped.

249 # 3. Results

250 ## 3.1. Mortality

251 At the normal application rate allowed in French apple orchards, chlorpyrifos-ethyl led to
252 high mortality in earwigs after 12 hours exposure, with marked differences depending on their origin
253 (Fig. 1). Earwigs collected in either organic or IPM orchards were more sensitive than earwigs
254 collected in conventional ones (p-value: 0.006 IPM-Conventional and 0.015 Organic-Conventional).
255 More than 80% mortality was observed in earwigs from organic and IPM, whereas less than 50%
256 mortality was observed in earwigs from the conventional orchard.

257 ## 3.2. Impact of chlorpyrifos-ethyl on metabolic pathways

258 Unexposed males and control females from conventional orchards exhibited about two times
259 higher basal CbE activity compared to earwigs from organic and IPM orchards (p-value < 0.005 for
260 both sexes). A four times higher level in unexposed constitutive GST in earwigs from conventional
261 orchards compared to organic and IPM ones was also observed (p-value < 0.005 for both sexes)
262 (Table 2; Fig 2).

263 After exposure to chlorpyrifos-ethyl, sex and earwig origin influenced the effect on CBEs
264 activity. We observed a strong decrease in CBEs in male earwigs caught either in organic or IPM
265 orchards (78.0% and 54.5% inhibition respectively). For females, only those collected in the organic
266 orchard showed a strong decrease in activity (-67.7%), with no significant difference observed for
267 females from the IPM orchard. Regarding the conventional orchard, a 29.9% decrease was observed
268 for females (p-value= 0.033), while no significant inhibition was recorded for males (p-value= 0.169)
269 (Fig. 2A & 2B).

270 In contrast, after exposure to chlorpyriphos-ethyl, GSTs activity increased, for both male and
271 female earwigs collected in IPM and conventional orchards (Fig. 2C & 2D). This increase was more
272 pronounced for females, especially for females collected in the IPM orchard (Fig. 2D). Concerning
273 earwigs collected in the organic orchard, despite their weak GSTs activity, we observed a significant
274 decrease in this enzyme activity for females, but no modification was observed for males.

275 ## 3.3. Impact of chlorpyrifos-ethyl on acetylcholinesterase activity

276 Basal AChE activity levels in both male and female earwigs are summarized in Table 2. No
277 significant differences could be observed either between sexes or management strategies. However,
278 exposure to chlorpyriphos-ethyl, induced a sex-dependent response in AChE activity (Fig. 3). Strong
279 and significant decreases in AChE activity were observed for males after chlorpyrifos-ethyl exposure
280 (80.3%, 45.4% and 73.4% inhibition for organic, IPM and conventional orchards respectively; p-

281 values= 0.003 for each strategy) while the female AChE remained constant (p-value= 0.878; 0.530
282 and 0.105 for organic, IPM and conventional strategies respectively).

283 *In vitro* inhibition kinetics in homogenates incubated in the presence of chlorpyrifos-ethyl-oxon
284 generated typical sigmoid dose-dependent curves (Fig. 4). The estimated IC_{50} values for the female
285 earwigs AChE were $7.83x10^{-8}$ M (organic); $9.30x10^{-8}$ M (IPM); $4.51x10^{-7}$ M (conventional). IC_{50} values
286 for the male earwigs were $1.30x10^{-7}$ M (organic); $8.68x10^{-8}$ M (IPM); 5.30 $x10^{-7}$ M (conventional).
287 Both sexes exhibited very closely related IC_{50} values. In order to compare AChE sensitivity between
288 strategies, a susceptibility ratio (SR) was calculated by dividing the conventional IC_{50} by organic or
289 IPM IC_{50}, for each sex independently. An increase in sensitivity compare to conventional organisms is
290 indicated by values greater than one (Table 3).

291 4. Discussion

292 There is increasing interest on understanding the underlying mechanisms that drive
293 evolutionary responses of populations to environmental changes including the role of plasticity and
294 resistance (Garnas, 2018). Insecticide resistance in non-target organisms reflects the long-term
295 negative effects of pesticides. It highlights the adaptive response and evolution of non-target
296 organisms subjected to anthropic pressure. A number of studies assessed the toxic impacts of
297 insecticides on pests but currently there is increasing interest in also assessing the impacts on
298 beneficial species, by describing the side-effects of pesticides with lethal and sub-lethal
299 consequences (Desneux et al., 2007; Biondi et al., 2012; Benamú et al., 2013; Schmidt-Jeffris and
300 Beers, 2018). In apple orchards, earwigs are an interesting beneficial arthropod. Based on the lethal
301 response data obtained in the present study, the 50% decrease in mortality of *F. auricularia* collected
302 in a conventional apple orchard (compared to organic or IPM orchards) suggests resistance to the
303 insecticide in this population. A similar decrease in mortality was previously observed in different
304 insect species such as pests (Aïzoun et al., 2013; Grigg-McGuffin et al., 2015) or natural enemies
305 (Tirello et al., 2012). Resistance to pesticides in effective biocontrol agents can be a desirable feature.
306 For example, the release of resistant populations of the predatory mite *K. aberrans* had been
307 successfully carried out in vineyards and apple orchards where pest control strategies included
308 chlorpyrifos and other pesticides (Duso et al., 2009, 2012; Ahmad et al., 2013). Here, we have
309 demonstrated for the first time that the earwig *F. auricularia* is able to acquire resistance to
310 chlorpyrifos insecticides.

311 Resistance to insecticides is known to depend on either changes in specific metabolic
312 pathways or mutation of the insecticide's molecular target. The response of two major enzyme
313 families commonly involved in xenobiotic detoxification including pesticides, GSTs and CbEs partially
314 supported our hypothesis that resistance could have arisen in the conventional orchard. First, a

315 higher constitutive activity of both GSTs and CBEs in earwigs collected in the conventional orchard
316 (compare to the organic one) is consistent with resistance in this species. We observed that basal
317 GST activity was 4-times higher in these earwigs compared to those from the organic orchard.
318 Previous studies have shown the involvement of GSTs in resistance to organophosphate insecticides
319 in the codling moth (Fuentes-Contreras et al., 2007; Voudouris et al., 2011). In the housefly *Musca*
320 *domestica,* GSTs activity was correlated with an increased level in mRNA and proteins, with a 2-4
321 times-higher level in resistant strains compared to susceptible ones (Fournier et al., 1992). Similar
322 effects were described for resistant versus susceptible strains of the diamondback moth *P. xylostella*
323 and the cockroach *B. germanica* resistant to chlorpyriphos-ethyl (respectivly a 2.2- and a 3.4-times
324 higher GSTs activities) (Yu and Nguyen, 1992; Valles and Yu, 1996); for the tufted apple bud moth *P.*
325 *idaeusalis* resistant to azinphosmetyl (2-times higher GSTs activity) (Carlini et al., 1995); and for the
326 red flour beetle *T. castaneum* and the mosquito *A. aegypti* resistant respectively to malathion and
327 temephos (5 and 4.6-fold higher GSTs activity) (Rodríguez et al., 2002). On another hand, in the
328 present study, we observed that GST activity was strongly induced, after exposure to chlorpyrifos-
329 ethyl in both pre-exposed (from conventional) and mid-exposed (from IPM) earwig populations. Such
330 an induction highlights the capacity of earwigs from those orchards to rapidly respond to OP
331 exposure, which agrees with high phenotypic plasticity in those individuals compare to the absence
332 of response for naïve earwigs (in organic orchard). Moreover, despite the low basal GST activity
333 observed in earwigs from the IPM orchard, the rapid induction of their GST activity reflects a positive
334 selection of individuals exhibiting efficient detoxification mechanism. The low basal GST activity
335 observed in earwigs from IPM orchard, suggests that when insecticide pressure was relaxed (end of
336 OP treatments since 2013) susceptible genotypes could have survived and reproduced themselves.
337 However, among this earwig population, a substantial amount of individuals were able to maintain
338 their ability to adapt to insecticide exposure. These two events confirm the plasticity of the
339 individuals belonging to the IPM population. They are consistent with the potential role of plasticity
340 in evolutionary response to pesticides depending on spatiotemporal variation in pesticide exposure
341 which can lead to rapid environmental changes (Cothran et al 2013; Hua et al. 2015).
342 The second metabolic pathway investigated involved CbE isoenzymes. As specified above,
343 constitutive CBEs activity in *F. auricularia* collected in the conventional orchard was two times higher
344 than that measured in the other earwigs. This result is similar to the quantitative increase observed
345 in the codling moth and the cotton aphid, where respectively a 2- and a 3.7-times higher constitutive
346 activity in resistant compare to susceptible strains was found (Pan et al., 2009; Reyes et al., 2011).
347 This enhanced production of CbEs confers resistance to insecticides, such as organophosphates,
348 carbamates, and to a lesser extent, pyrethroids by sequestering and metabolizing these insecticides
349 before they reach the nervous system (Bass et al., 2014). A quantitative increase in CBEs activity has

350 been shown to induce broader metabolic resistance compared to qualitative modification (Cui et al.,
351 2015). In this case, the protection results in an effective molecular sequestration of OP insecticides
352 (Sogorb and Vilanova, 2002; Wheelock et al., 2008), which avoids inhibition of AChE, the molecular
353 target of OP. This protective mechanism has been described in numerous cases including aphids (Sun
354 et al., 2005; Bass et al., 2014) and mosquitoes (Mouches et al., 1986; Hopkins et al., 2017).

355 We observed a decrease in male AChE activity after exposure to chlorpyriphos-ethyl. The
356 inhibition rate was similar in earwigs from the conventional and organic orchards but slightly
357 different in those from the IPM orchard. However lethal responses suggested that earwigs from the
358 conventional orchard were able to best survive the chlorpyrifos-ethyl exposure. Therefore, we
359 performed an *in vitro* inhibition trial with chlorpyrifos-oxon to elucidate whether the higher
360 susceptibility of earwigs from the organic orchard, could be explained by a higher affinity of AChE for
361 chlorpyrifos-oxon. The AChE IC50 values of males and females from organic and IPM were lower than
362 those from conventional orchards (4- and 5.5-times respectively). Therefore, the intrinsic sensitivity
363 of AChE to this OP insecticide could be a significant additional explanation for the differences
364 observed between management practices. AChE activity and sensitivity support the ability of earwigs
365 collected in conventional orchards to better cope with chlorpyrifos exposure. In addition, our
366 findings highlight the fact that higher resistance levels could be reached when an increase in CBEs
367 activity was associated with a modified and less sensitive form of AChE. It is generally accepted that
368 modified AChE leads to a specific insensitivity to OPs as demonstrated in Hemiptera, Diptera and
369 mites (Russell et al., 2004; Gilbert and Gill, 2010), but in some cases, no corresponding mutations
370 could be found in partial gene sequences from resistant insects (Javed et al., 2003). Deeper analysis
371 to evaluate *F. auricularia* AChE susceptibility is needed to better allocate which is due to tolerance
372 and resistance mechanisms.

373 In addition to earwig origin, AChE inhibition after chlorpyrifos exposure depended on sex. In
374 recent studies, we have shown that *F. auricularia* males are more sensitive to OP insecticides than
375 females using both biochemical biomarkers (Malagnoux et al., 2014) and predation activity
376 (Malagnoux et al., 2015b). The LD_{50} observed for chlorpyrifos-ethyl and acetamiprid on *F. auricularia*
377 confirmed that males are slightly but significantly more sensitive than females (Malagnoux et al.,
378 2015a). The *in vivo* response of AChE to chlorpyrifos-ethyl exposure observed in the present study,
379 clearly support the differential responses of males and females in this earwig species. These results
380 point out the need to analyze both sexes independently, to better assess the impact of insecticides
381 on this species either from a biochemical or physiological point of view.

382 Insecticide resistance is frequently associated with fitness costs, explaining why susceptible
383 genotypes increase in frequency when insecticide pressure is relaxed (Foster et al 2017). It is well
384 known that reallocation of energy reserves to maintain homeostasis under pesticide exposure mainly

385 focuses on protein induction to improve defense mechanisms, which often involves overproduction
386 of detoxification enzymes (Calow, 1991; Kooijman, 2010). Earwigs from conventional orchards
387 increase detoxification metabolism and divert energy from maintenance and growth. Indeed,
388 *F. auricularia* weighed less and had smaller morphometric traits (inter-eye distance, femur length) in
389 conventional than organic orchards (Suchail et al., 2018) which is consistent with the reallocation of
390 reserves to current induction of GSTs and higher CbE levels.

391 In conclusion, this study is the first report of *F. auricularia* insecticide tolerance. Our results
392 underline the differences in metabolic pathway responses in earwig populations influenced by their
393 historic management strategies. In particular, constitutive GSTs and CBEs activity levels were higher,
394 and a decrease in sensitivity of AChE was observed in male and female earwigs pre-exposed in the
395 conventional orchard. After exposure the absence of AChE inhibition in females suggests that, *in vivo,*
396 further mechanisms could be involved in this sex. Other biological parameters should additionally be
397 investigated and extended to higher amount of commercial orchards to fully understand
398 relationships between insecticide use and susceptibility, and define how agricultural strategies
399 influence earwig life-history traits. Overall our findings highlight the need to develop an integrated
400 approach to assess both the physiological and biochemical modifications induced by pest
401 management strategies on natural enemies.
402

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624 the diamondback moth. Pestic. Biochem. Physiol. 44, 74–81.
625
626

627 Table 1. Origin of earwig populations analyzed and the average of their received treatments
628 regarding OPs and total neurotoxic insecticides in the last 10 years. Neurotoxic insecticides include
629 OP, neonicotinoid and synthetic pyrethroid compounds.
630

Earwig population	Origin	OP insecticides per year	Neurotoxic insecticides per year
Organic	Organic commercial apple orchard using « Alt'Carpo » netting system	0	0
IPM	Commercial apple orchard under IPM strategy	1	6
Conv	Commercial apple orchard under conventional strategy	5,9	11,4

631
632
633 Table 2. Specific enzyme activities, expressed as U.mg-1 protein (mean±SD) were measured
634 in homogenates from the head (AChE) or abdomen (GSTs and CbE) of *Forficula auricularia* earwigs,
635 sampled from three different management strategies.

	Acetylcholinesterase ($mU.mg^{-1}$)			Carboxylesterase ($U.mg^{-1}$)			Glutathion-S-transferase ($U.mg^{-1}$)		
	Organic	IPM	Conv	Organic	IPM	Conv	Organic	IPM	Conv
Male	40.86 ± 17.68	40.81 ± 15.01	32.63 ± 12.24	1.01 ± 0.29	1.03 ± 0.44	1.86 ± 0.69	1.77 ± 0.56	2.8 ± 1.92	9.18 ± 4.57
Female	30.55 ± 16.39	26.35 ± 12.98	28.62 ± 13.49	0.69 ± 0.31	0.92 ± 0.59	1.52 ± 0.66	1.98 ± 1.16	4.24 ± 3.31	8.83 ± 3.83

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639 Table 3. Susceptibility ratio (SR) for *in vitro* inhibition kinetics of AChE incubated in the
640 presence of chlorpyrifos-ethyl-oxon (10^{-12} to 10^{-3} M final concentration) for 30 min at 25°C. The SR is
641 calculated by dividing the conventional IC_{50} by the organic or IPM IC_{50}, for each sex independently. A
642 value greater than 1 indicates increased sensitivity.

	SR values for males	SR values for females
Organic	4.11	5.76
IPM	6.11	4.85

643

644 Figure legends

645 Figure 1: Effect of chlorpyrifos-ethyl exposure (12h, in a climate-controlled chamber at 20°C
646 and subjected to variations in natural light) on *F. auricularia* mortality (%). Tukey box plots indicate
647 the median, 25th and 75th percentiles (box edges), the range (whiskers) and outliers (black dots).
648 Different letters denote significant differences between management strategies (p-value: 0.003).

649

650 Figure 2: Response of Carboxylesterase (CbE) and Glutathion-S-Transferase (GST) activities
651 ($U.mg^{-1}$ proteins) in earwigs depending on management strategy after 12h exposure to chlorpyrifos-
652 ethyl. Tukey box plots indicate the median, the 25th and 75th percentiles (box edges), the range
653 (whiskers) and outliers (black dots). Different letters indicate significant differences between
654 orchards and treatment (the CbEs p-values for male and female between orchard and treatment
655 were less than 0.001 (same results for GSTs). Activities were measured on extracts from the
656 abdomen of the earwig *Forficula auricularia*. For each experiment n=20, except for the earwigs
657 collected in organic orchards after chlorpyrifos-ethyl exposure where n=6.

658

659 Figure 3: Response of Acetylcholinesterase (AChE) activity in earwig males (A) and females
660 (B) depending on management strategy after 12h exposure to chlorpyrifos-ethyl. Tukey box plots
661 indicate the median, 25th and 75th percentiles (box edges), the range (whiskers) and outliers (black
662 dots). Significant differences between orchards and treatment are indicated by different letters
663 (females p-value for treatment = 0.08; female p-value for orchard = 0.19; male p-value <0.001 for
664 both factors). For each experiment n=20, except for the earwigs collected in organic orchards after
665 chlorpyrifos-ethyl exposure where n=6.

666

667 Figure 4: Dose-dependent inhibition curves for *Forficula auricularia* acetylcholinesterase
668 (AChE) in the presence of serial molar concentrations of chlorpyrifos-ethyl-oxon. Symbols are the
669 mean±SD expressed as percentage of the control. Each point corresponds to the mean of three
670 independent assays.

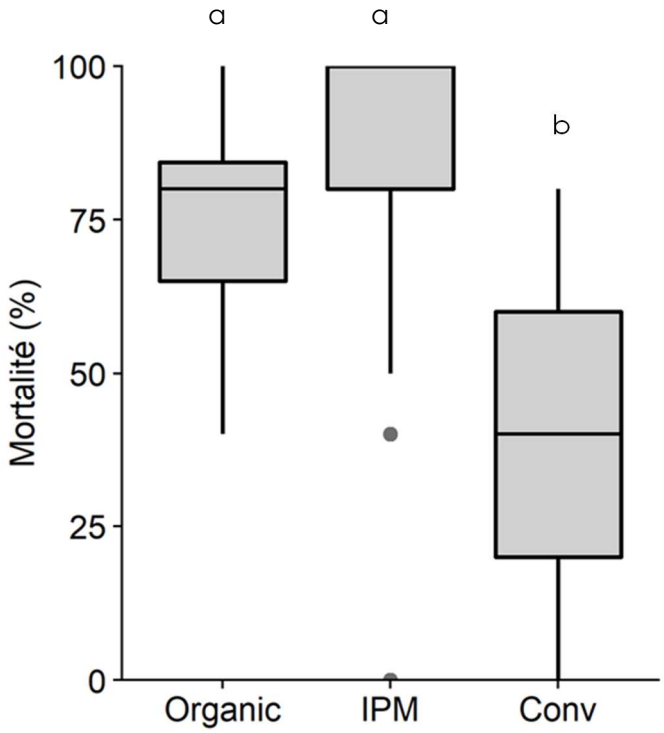
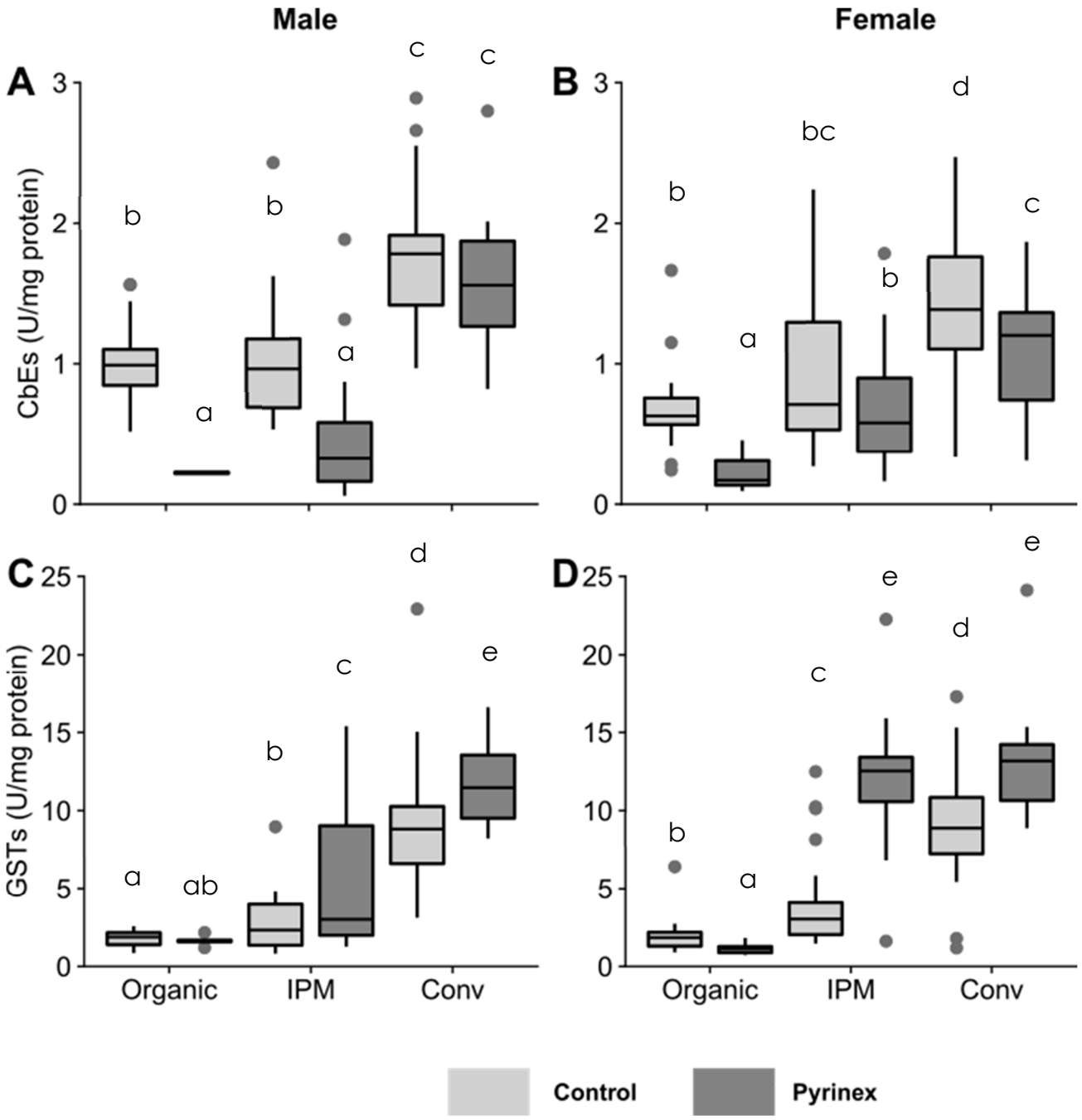
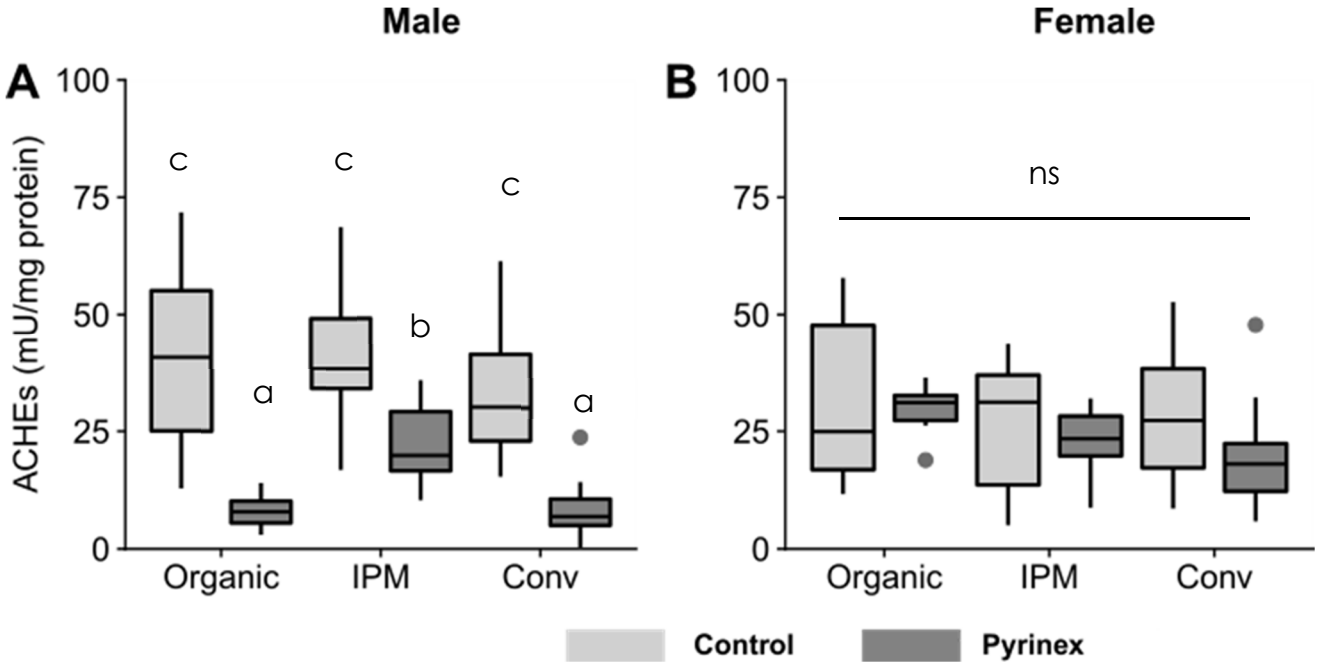


Fig 1

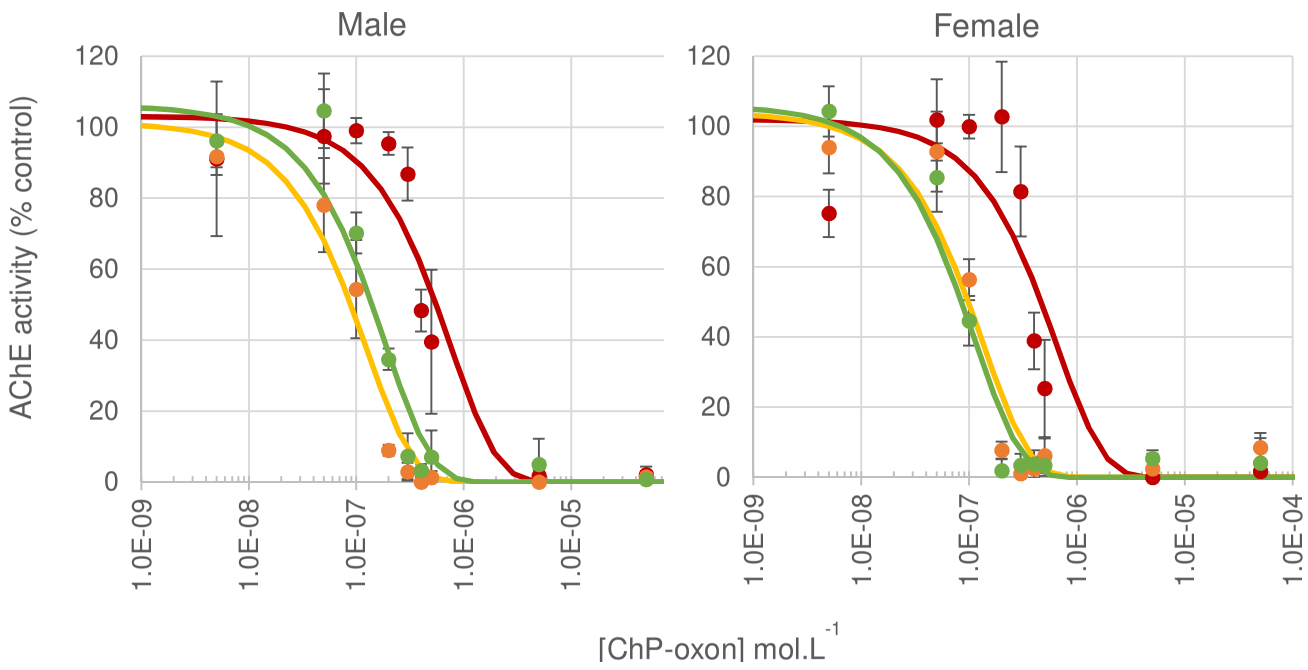

Male
Female
A
B
C
D
CbEs (U/mg protein)
GSTs (U/mg protein)
Organic
IPM
Conv
Control
Pyrinex

673 Fig 2

674


Male
Female
A
B
ACHEs (mU/mg protein)
100
75
50
25
0
c
a
c
b
c
a
ns
Organic
IPM
Conv
Control
Pyrinex

675 Fig 3.


Male
Female
AChE activity (% control)
120
100
80
60
40
20
0
1.0E-09
1.0E-08
1.0E-07
1.0E-06
1.0E-05
1.0E-04
[ChP-oxon] mol.L-1

676 Fig 4