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# Biochemical and Molecular Mechanisms Associated With the Resistance of the European Corn Borer (Lepidoptera: Crambidae) to Lambda-Cyhalothrin and First Monitoring Tool

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13 **Biochemical and molecular mechanisms associated with the resistance of**  
14 **the European corn borer (*Ostrinia nubilalis*) to lambda-cyhalothrin and**  
15 **first monitoring tool**

16  
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25

## Abstract

27 The European corn borer (*Ostrinia nubilalis*) is one of the most serious corn pest in Europe  
28 where it is controlled with pesticides, in particular, pyrethroids. First control failures with this  
29 chemical family occurred on the field in 2008 in the centre of France, and the first resistance  
30 case was described in 2012. In the present study, we investigate resistance mechanisms  
31 involved in seven French populations of *O. nubilalis* collected in the field.

32 Resistances to deltamethrin and lambda-cyhalothrin were confirmed with a higher resistance  
33 ratio for lambda-cyhalothrin (63.79 compared to 7.67). Resistance to the two active compounds  
34 was correlated except for one population, indicating a high probability of cross-resistance.  
35 Analyses of the activity of three major families of detoxification enzymes in resistant  
36 individuals showed a significant increase of the average MFO activity in males of four  
37 populations (activity ratios of 2.76 to 5.73) and higher GST activity in females of two other  
38 populations (activity ratios 4.48 and 5.21). Molecular investigation of the sodium channel gene  
39 sequence showed the presence of the kdr mutation in a highly resistant individual. We designed  
40 a PCR-RFLP screening tool to search for this mutation in the field, and we found it in five  
41 populations but not in the susceptible one.

42 The resistance of *O. nubilalis* to pyrethroids in France seems to result from a combination of  
43 resistance mechanisms, possibly as a consequence of a selection pressure with an exceptional  
44 duration (almost 40 years old).

45 **Keywords:** pyrethroid, deltamethrin, kdr, cytochrome p450 (MFO), glutathione-S-transferase  
46 (GST), carboxylesterase (EST), detoxification enzymes, target mutation.

48

49 The control of insect pests is a major issue in agriculture. However, management strategies  
50 relying on insecticides may be compromised by pest insecticide resistance. Until now there  
51 have been no reports of resistance to chemical insecticides for the European corn borer  
52 (*Ostrinia nubilalis*) (Pyraloidea, Crambidae, Pyraustinae) despite the fact that it is one of the  
53 most important corn pest in Europe and North America (Bohnenblust et al. 2014). This  
54 polyphagous moth is native from northern Africa, western Asia, and Europe (Hudon et al.  
55 1989) and was accidentally introduced into North America at the beginning of the twentieth  
56 century (Caffrey and Worthley 1927). The larvae bore into stalks and ears of corn. In France,  
57 the loss was evaluated on average at 4 % of yield potential per larvae per plant. Losses can be  
58 higher in the case of water stress (Thibord 2009). For the last 15 years, control of *O. nubilalis*  
59 has been widely based on the use of transgenic Bt corn in America and numerous studies have  
60 investigated mechanisms of resistance to Bt toxins (Bravo et al. 2011) and resistance  
61 management schemes (Huang et al. 2011). The emergence of insects resistant to Bt corn in  
62 the field has been slow, possibly because the resistance alleles were initially rare (frequency  
63 <1 ‰) (Bourguet et al. 2003, Stodola et al. 2006) and strategies have been designed to slow  
64 down the evolution of resistance (Huang et al. 2011).

65 Control of *O. nubilalis* is based on the use of insecticides in regions of the world where GM  
66 crops are not grown, for example, in France. In this country, the insecticide based control of  
67 this pest where is mostly performed using pyrethroids since late 1970's. The first evidence of  
68 possible resistance to pyrethroids in *O. nubilabis* was reported in the mid-2000s in central  
69 France. Between 2008 and 2011 field experiments in this area confirmed a low efficiency of  
70 lambda-cyhalothrin and other active ingredients belonging to the family of pyrethroids  
71 (Thibord, 2009 pers. Com.). Resistance to pyrethroids was formally reported in 2012 when  
72 bioassays on insects collected in fields of this same region with excessive damage showed

73 resistance ratio as high as 119 in the laboratory (Siegwart et al. 2012). To our knowledge, such  
74 resistance was not reported elsewhere in this species. In the present study, we aimed to  
75 characterise the observed resistance better and to determine underlying mechanisms.

76 Pyrethroids are neurotoxic to insects and act by binding on the voltage-gated sodium channel.  
77 Resistance to pyrethroids is reported in numerous insect pests (Soderlund and Knipple 2003).  
78 Two main categories of mechanisms have been reported to cause resistance. The first is  
79 associated with the detoxification of the insecticide molecule by specific enzymes (Liu and  
80 Scott 1998, Kranthi et al. 2001). The second is linked to non-synonymous mutations in the gene  
81 coding for the voltage-gated sodium channel, the principal site of pyrethroid action. Mainly two  
82 mutations have been found in numerous pests, the first, named *kdr* for ‘knock down resistance’  
83 was the first reported in 1951 and is the most common one (Busvine 1951, Soderlund and  
84 Knipple 2003). The second mutation, *super-kdr*, was reported 16 years later and was also found  
85 in various insect pests (Farnham 1977, Soderlund and Knipple 2003). Detoxification and  
86 mutation are not exclusive, and both types of resistance can coexist in a single insect population  
87 (Franck et al. 2007).

88 In the present study, we chose to assess the presence of these two types of resistance  
89 mechanisms in French populations of *O. nubilalis* collected in corn fields where resistance was  
90 either suspected or not. (i) First, we assessed if resistance was associated with increased  
91 detoxification by specialised enzymes by measuring activities of three major families:  
92 Oxygenases P<sub>450</sub> (MFO), the glutathione-S-transferases (GST) and carboxylesterase (EST). (ii)  
93 Second, we assessed if the resistance was acquired through a mutation of the molecular target  
94 gene of pyrethroids: the voltage-gated sodium ion channel by sequencing a part of this gene  
95 carrying *kdr* and *super kdr* mutations in domains IIS4, IIS5 and IIS6 segments.

96

97

99 **Insect sampling**

100 We used as a reference one susceptible laboratory strain that has been mass reared on an  
101 artificial diet at the INRA of Magneraud (France) for 20 years without insecticide selection  
102 pressure. This strain originated from a non-treated field near Paris (France). Other individuals  
103 were collected as diapausing larvae directly in corn stalks in 7 corn fields located near the  
104 following cities (French department number): Epieds-en-beauce (45); Semerville (41);  
105 Tripleville (41); Labosse (41); Binas (41) Montardon (64) and Rouffach (68). Larvae were  
106 stored four months at 4 °C in the laboratory to break down diapause. The larvae were then  
107 individualised in small boxes with some moist cotton in a climatic chamber at 23 °C and 16:8  
108 h L:D photoperiod. Adult emergence was checked every day. As soon as we obtained one male  
109 and one female from the same population, these were paired for reproduction. We thus assess  
110 16 pairs for Epied en Beauce; 14 pairs for Semerville; 3 pairs for Labosse; 3 pairs for Binas; 12  
111 pairs for Montardon and seven pairs for Rouffach.

112 Offsprings were submitted to toxicological tests at the neonatal stage (bioassays). Individuals  
113 that survived the bioassay at high insecticide concentration (up to 13 ppm for lambda-  
114 cyhalothrin or 13.28 ppm for deltamethrin, see below) were kept in individual vials containing  
115 artificial diet without insecticide pressure during the rest of their life cycle. As soon as these  
116 resistant individuals emerged as adults, half of their abdomens were dissected and immediately  
117 used to measure MFO activity; protein extractions were performed on the second half for GST  
118 and EST activities measurement, and the rest of the insect body was individually kept in 90 %  
119 ethanol at 4 °C for genetic analyses.

120 Following the bioassay results, we selected and sequenced a highly resistant individual from  
121 Semerville (n°205) and a non-treated from Montardon (n°3) to seek for the *kdr* and *Super-Kdr*  
122 mutations. Further, we selected 37 individuals to create a molecular *kdr* detection tool. This

123 selection include 26 individuals that are survivors to very high insecticide concentration, plus  
124 six from the location Rouffach (where the kdr mutation was first detected), plus five samples  
125 from the non-treated population of Montardon.

126

## 127 **Insecticides**

128 The commercial formulations of lambda-cyhalothrin (Karate Zeon) and deltamethrin (Decis  
129 Protech) were purchased from Syngenta (France) and Bayer (France). The insecticides were  
130 diluted in osmosed water. Insecticide ranges used to test the reference strain were 0.54 ; 1.17 ;  
131 1.8 ; 3.9 ; 6 and 9.5 ppm for lambda-cyhalothrin and 2.61 ; 3.91 ; 5.88 ; 8.84 ; 13.28 ; 19.96  
132 and 30 ppm for deltamethrin. These concentrations were adapted for field populations (see  
133 below).

134

## 135 **Bioassays**

136 Susceptibilities of the reference strain and offspring of wild individuals were evaluated by  
137 bioassays on the neonate larvae stage. Microplate (96-wells, Sterilin Ltd, Newport, UK) wells  
138 are first filled with 150  $\mu$ L of artificial diet (Stonefly Industries Ltd, Rochester, NY). Six  $\mu$ L  
139 of insecticide solution was overlaid on the diet, and one neonate larva (0-4h old) was deposited  
140 on each well 20 min after treatment (Reyes and Sauphanor 2008). Controls were placed on each  
141 plate on, untreated artificial diet, to estimate the percentage of natural mortality. Osmosed water  
142 replaced insecticide for the controls (Fuentes-Contreras et al. 2007). Wells were sealed with  
143 parafilm and a lid was placed on each microplate to prevent larvae escape. Mortality was  
144 recorded after seven days at 23 °C. A larva was considered as dead when not responding to a  
145 probe with dissecting forceps. Missing larvae were subtracted from the initial number.

146 The corrected mortality was calculated using the Abbott formula (Abbott 1925).

147 For each insecticide concentration, the average sensitivity level of a population/strain was  
148 calculated as the mean corrected mortality of all individuals from this population/strain.

149 For each population/strain, concentration-response relationships were established per  
150 insecticide. The six concentrations of lambda-cyhalothrin and seven of deltamethrin (see above)  
151 giving between 0 and 100 % mortality for the reference strain were initially used. When a  
152 population contained resistant individuals, i.e. individuals that survived the highest  
153 concentrations tested on the reference strain (9.5 ppm of lambda-cyhalothrin or 30 ppm of  
154 deltamethrin), extra upper concentrations were tested. These concentrations are chosen by  
155 keeping the same multiplying factor used in the original range until we reached 100 % of  
156 mortality. Probit analyses were performed on these results with the free software priprobit 1.63  
157 (Sakuma 1998); <http://ars.usda.gov/Services/docs.htm?docid=11284>). All resistance ratios  
158 were calculated on the basis of these analyses.

### 159 **Enzymatic activities**

160 The general principle for the dosage of enzymatic activities is to measure the quantity of the  
161 product of an enzymatic reaction and to divide it by the total protein content of the sample. The  
162 amount of product is determined by a level of absorbance or fluorescence. MFO activity was  
163 measured on fresh half posterior parts of adult abdomens directly placed in the reaction solution.  
164 Measurement of GST and EST activities were performed using the second (anterior) half of the  
165 abdomen and necessitated a preliminary phase of protein extraction.

166 The total protein content of each sample was measured with Bradford colorimetric test using  
167 bovine serum albumin to build the standard curve (Bradford 1976).

168 Fluorescence and absorbance were measured using a microplate reader (Infinite 200, Tecan,  
169 Männedorf, Switzerland).

### 170 *Mixed-function oxidase (MFO)*

171 The MFO activity was determined using 7-ethoxycoumarin O-deethylation (ECOD) (Ulrich  
172 and Weber 1972) adapted for in vivo analysis in microplates. Adult abdomens were dissected  
173 and directly homogenised in an incubation solution containing 100 µl of Hepes buffer (50 mM



174 pH 7) with 7-ethoxycoumarin (0.4 mM) on the ice. After 4h incubation at 30 °C, the reaction  
175 was stopped by adding 100 µL of 1.5M glycine buffer (pH 10.3) and centrifuged at 15 000 × g  
176 for 2 min at 2 °C. Supernatants were individually placed in wells of black microplates (96-  
177 wells, Corning Costar®, New York, U.S). The 7-hydroxycoumarin (HC) fluorescence was  
178 quantified with 380 nm excitation and 465 nm emission filters. Three wells per microplates  
179 were left without samples but just the mix receiving glycine buffer previous to incubation to  
180 have a blank. Protein dosages were made on this reaction product after fluorescence  
181 measurement. A 10 fold dilution was operated before this dosage using the Bradford method  
182 (Bradford 1976). The MFO activity was expressed as pg of 7-HC/µg of total protein/min by  
183 using a standard curve of 7-Hydroxycoumarine (HC) (0.5-4.5 nmoles/well) to convert  
184 fluorescence in 7 HC quantity.

#### 185 *Glutathione S-transferase (GST)*

186 For protein extraction, anterior half abdomens were crushed in 110 µl of Hepes buffer (50 mM,  
187 pH 7) on the ice and the obtained homogenates were centrifuged at 15 000 g for 15 min at 4 °C.  
188 Supernatants were stored at -80 °C before use. When all extractions were finished, the  
189 supernatants were used as enzyme sources for reactions in a single test to limit handling errors  
190 (Bouvier et al. 2002). GST activity was determined in transparent microplates (96-wells,  
191 Sterilin®, Newport, UK) using 2,4-dinitro-chlorobenzene (CDNB) as substrate (Nauen and  
192 Stumpf 2002). The reaction mixture in one well consisted of 2 µl of enzymatic extract, 198 µl  
193 of a solution containing: 10 µl of 50 mM glutathione (GSH), 185 µl of Hepes buffer (50 mM,  
194 pH 7.0) and 3 µL of 50 mM CDNB. Three wells per microplate were filled with 2 µl of Hepes  
195 buffer (50 mM, pH 7.0) instead of enzyme extract as blank. Absorbance was measured after 2  
196 min of incubation at 25 °C in kinetic mode every 30 seconds at 340nm. Since the CDNB-  
197 glutathione adduct was not commercially available, we were unable to build a standard curve,  
198 so we used the molar extinction coefficient ( $9.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ ) of CDNB-glutathione to convert

199 absorbance in  $\mu\text{mol}$  of CDNB-glutathione. The final specific activity was expressed in  $\mu\text{mol}$  of  
200 CDNB-glutathione/min/mg of total protein extracted.

#### 201 *Carboxylesterases (EST)*

202 The same protein extracts were used as for GST. Total non-specific EST activity was measured  
203 with  $\alpha$ -naphthyl acetate ( $\alpha$ -NA) as substrate (Reyes 2007). The reaction mixture was 1  $\mu\text{l}$  of  
204 protein extract and 194  $\mu\text{l}$  of 30  $\mu\text{M}$   $\alpha$ -NA in HEPES buffer (50 mM, pH 7.0) in each microplate  
205 well. After 20 min of incubation at 22 °C in darkness, the reaction was stopped and coloured  
206 by adding 55  $\mu\text{l}$  of 0.2 % Fast Garnet GBC diluted in 2.5 % sodium dodecyl sulphate solution.  
207 Absorbance was recorded at 590 nm, after incubation for 20 min in darkness at room  
208 temperature.

209 The standard curve with  $\alpha$ -Naphthol (0-18 nmoles/well) was elaborated to express activity in  
210 nmoles of product/min/mg of total proteins.

#### 211 **Sequencing and detecting the *kdr* mutation**

212 For genetics studies, total DNA was individually extracted from the head of adults moths  
213 following Wash *et al.* (Walsh et al. 1991) with 100  $\mu\text{l}$  of 10 % Chelex 100 (Biorad) solution  
214 and 3  $\mu\text{l}$  (10 mg/ml) of proteinase K (Eurobio). Tissues were digested overnight at 56 °C. After  
215 boiling for 30 min, supernatants were used as DNA templates for PCR reaction. Sequencing  
216 was done on two selected individuals and detection on 37 moths (see insect sampling).

#### 217 *Sequencing the *kdr* mutation*

218 The design of all primers was made using the Primer 3 web interface version 0-4-0  
219 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>)(Rozen and Skaletsky 1999). As no sequence was  
220 available for *O. nubilalis* in this part of the genome, we designed our primer OsNa-R2 matching  
221 the closest phylogenetic sequence available on GenBank: *Cnaphalocrocis medinalis*  
222 (Pyraloidea, Crambidae, Spilomelinae - GenBank accession KC342959 & AGH70334) for the  
223 detection of the *kdr* and super*kdr* mutations. According to the high similarities between *Cydia*

224 *pomonella* and *C. medinalis* we used primers SKdr-F and SKdr-R, previously designed in our  
225 lab for this purpose on *C. pomonella* (Franck et al. 2012) as PCR and sequencing primers for  
226 the detection of the *Super-Kdr* mutation (Table 1). We then amplified the whole region carrying  
227 both mutations (Figure 2) using SKdr-F and OsNa-R2 at low annealing temperature on two  
228 selected individuals.

229 PCR amplifications were performed with a Mastercycler thermocycler (Eppendorf). Each  
230 reaction had a volume of 25 µl containing the following MIX : 1X GoTaq® Flexi Buffer, 1.5  
231 mM MgCl<sub>2</sub>, and 0.1 mg/ml Bovine Serum Albumin, 200 µM of each dNTPs, 0.4 µM of each  
232 Forward and Reverse primers, 1 unit of GoTaq® Flexi DNA Polymerase (Promega) and 4 µl  
233 of DNA template. The PCR conditions were: 3 min at 95 °C followed by 35 cycles at 95 °C for  
234 45s, 50 °C for 1 min, and 72 °C of elongation for 1.5 min with a final extension step at 72 °C  
235 for 10 min.

236 An approximately 1400pb PCR product was obtained and sequenced (GATC Biotech) with  
237 PCR primers. Sequences were manually edited and aligned both in forward and reverse  
238 directions and assembled using the Bioedit Software (Hall 1999). We verified the sequence  
239 homology with voltage-gated sodium ion channel sequences known in other susceptible and  
240 resistant Lepidoptera (*Bombyx mori*, *Plutella xylostella*, *Cydia pomonella*) and validated the  
241 presence of the Kdr L1014F mutation. The obtained complete sequences were deposited in the  
242 GenBank database (accession N° KU588158 for the resistant individual and KU588159 for the  
243 susceptible one), incomplete or poor quality sequences were not deposited.

#### 244 *Detection of the kdr mutation*

245 To screen the kdr mutation in the 37 selected individuals, we used a slightly modified nested  
246 PCR approach described by Schuelke (2000) coupled with a PCR-RFLP method from Franck  
247 et al. (2007). We designed a new specific primer pair (all primers detailed in Table 1): Forward Os-  
248 Kdr1 and reverse Os-Kdr2. Os-Kdr1 was conjugated with a universal 5'-

249 GTTGTAAAACGACGGCCAGT-3' M13-Tail; at its 5' end, the same universal 6-FAM (6-  
250 carboxyfluorescein) labelled M13-Tail was used for fluorescence detection. We used a ratio of  
251 1 M13-tailed-Os-Kdr1 primer for 10 Labelled universal M13-tail and 10 Os-Kdr2 reverse  
252 primers. We found two size of amplicon, probably due to intron variability: 268 and 273 bp.  
253 PCR amplifications were carried out with a Mastercycler thermocycler (Eppendorf). The  
254 reaction occurred in 12 µl containing : 1X GoTaq® Flexi Buffer, 1.5 mM MgCl<sub>2</sub>, 0.1 mg/ml  
255 Bovine Serum Albumin, 200 µM of each dNTPs, 0.4 µM of each labelled M13-Tail and specific  
256 reverse primer, 0.04 µM of M13-Tailed specific forward primer, 1 unit of GoTaq® Flexi DNA  
257 Polymerase (Promega) and 2 µl of DNA template. The PCR conditions were: 3 min at 95 °C  
258 followed by 30 cycles at 95 °C for 30 s, 54 °C for 45 s, 72 °C for 45s and 10 cycles at 95 °C  
259 for 30s, 50 °C for 45 s, 72 °C for 45s with a final extension step at 72 °C for 20 min.

260 All the PCR products were digested at 65 °C for 16 hours with two units of Tas I endonuclease  
261 and 1X of Buffer N°B (Fermentas) in 20 µl of final reaction volume. Ten µl of digested products  
262 were diluted in 100 µl H<sub>2</sub>O; 2 µl of this dilution, 7.8 µl of HiDi formamide, and 0.2 µl  
263 GeneScan™- 600 LIZ® Size standard (Applied Biosystems) were injected on an ABI 3730xl  
264 DNA Analyzer (Applied Biosystems) using POP7 polymer. These genotyping runs were  
265 analysed using GeneMapper® V4.1 Analysis Software (Applied Biosystems).

266 Each revealed fragment length was defined as an allele.

### 267 **Statistical analyses**

268 A probit analysis on corrected mortalities (Abbott 1925) was performed to determine the LC50  
269 / LC90 values (Raymond 1985). Resistance ratios at the LC50 (RR50, which is the ratio  
270 between the LC50 of the resistant population and the LC50 of the reference strain) and their 95  
271 % CI were calculated. The LC50s were considered different when the 95 % CI of their RR50  
272 did not include 1 (Robertson et al. 2007). Correlation between resistance to lambda-cyhalothrin  
273 and deltamethrin was performed with the Pearson method. Enzymatic activities data were

274 subjected to the non-parametric analysis of variance (Kruskal-Wallis test) because the data  
275 deviated for normality. Means were pairwise compared by the protected least significant  
276 difference Wilcoxon test ( $P < 0.05$ ) using the software R (Bell Laboratories, Murray Hill, US).

277

## 278 **Results**

### 279 **Toxicological tests**

280 There was a wide diversity of susceptibility to lambda-cyhalothrin in the field as shown by the  
281 continuous range of concentration-mortality response curves in toxicological tests (Figure 1).

282 All field populations showed a lower susceptibility to lambda-cyhalothrin than the reference  
283 strain. Resistance ratios at LC50 varied from 2.04 to 63.79 for the less (Montardon) to the most  
284 resistant population (Epieds-en-Beauce) (Table 2).

285 Resistance ratios to deltamethrin were systematically lower at LC50 than for the lambda-  
286 cyhalothrin. Despite this, 4 out of 7 populations had significantly higher LC90 than the  
287 reference strain (Table 2).

288 The resistance ratios to lambda-cyhalothrin and deltamethrin were strongly correlated among  
289 populations ( $r=0.93$   $p=0.007$ ) if the population Epieds-en-Beauce was removed from the  
290 analysis. Numerous individuals from Epieds-en-Beauce were resistant to lambda-cyhalothrin,  
291 but few were resistant to deltamethrin.

### 292 **Enzymatic activities**

293 The GST activities were assessed in only five populations owing to the mortality induced by  
294 toxicological tests in populations Rouffach and Montardon. Activity differences were evident  
295 between sexes with males showing higher activity levels (Kruskal-Wallis  $\chi^2= 14.6$ ,  $df = 1$ ,  $p =$   
296  $1.3 \cdot 10^{-4}$ ). A large inter-population heterogeneity of GST specific activity was observed (Table  
297 3). Mean GST activity of females from wild populations was 5.21 to 3.24 times higher than in  
298 the reference strain, but only females of the Semerville population had significantly higher GST

299 activities than the reference (Kruskal-Wallis  $\chi^2 = 20.7$ ,  $df = 1$ ,  $p = 5.1 \cdot 10^{-6}$ ). Activity ratio  
300 average 2 for males, and no significant difference when compared to the reference strain.

301 The specific activities of carboxylesterases (EST) were assessed in the same five populations  
302 (Table 3) as the GST. Differences were observed between sexes (Kruskal-Wallis  $\chi^2 = 46.3$ ,  $df =$   
303  $1$ ,  $p = 9.8 \cdot 10^{-12}$ ). No significant differences in activity were observed with the reference strain,  
304 considering males and females separately or not, but the three more resistant populations had  
305 the highest activities (ratios up to 2). For example between the reference strain and the most  
306 resistant population: Epied-en-Beauce for males the Kruskal-Wallis  $\chi^2 = 3.7$ ,  $df = 1$ ,  $p = 0.053$   
307 and for females the Kruskal-Wallis  $\chi^2 = 3.6$ ,  $df = 1$ ,  $p = 0.058$ .

308 The MFO activities were assessed in six populations (Table 3). As for GST, there was a  
309 difference in specific activity between sexes (Kruskal-Wallis  $\chi^2 = 138.5$ ,  $df = 1$ ,  $p < 2.2 \cdot 10^{-16}$ ).  
310 Males exhibited higher activity values than females. MFO activities differed between  
311 populations (Kruskal-Wallis  $\chi^2 = 104.9$ ,  $df = 13$ ,  $p < 2.2e-16$ ). Males of four populations  
312 exhibited higher MFO specific activity than the reference strain while in the two other  
313 populations (Semerville and Labosse) females had significantly lower activities than the  
314 reference strain or any other population.

### 315 **Genetic analyses**

316 Alignment of sequences of the susceptible and the resistant individual highlighted 17 mutations  
317 and deletions in the sodium channel gene  $\alpha$ -subunit carrying *kdr* and super *kdr* mutations loci.  
318 They were all located in introns except two: one silent mutation (V991) and the *kdr* mutation  
319 (Figure 2). This latter mutation is identical to that in *C. pomonella* and other pests or human  
320 disease insect vectors (Soderlund and Knipple 2003, Brun-Barale et al. 2005). At the protein  
321 level, it leads to the replacement of a Leucine in position 1014 to a Phenylalanine The position  
322 number was attributed according to the amino acid sequence of the most abundant splice variant  
323 of the house fly *Vssc 1* sodium channel protein (Ingles et al. 1996, Williamson et al. 1996). We

324 have not found the super kdr mutation in the resistant individual sequenced.  
325 The PCR-RFLP detection tool gave clear results (Supplementary Figure 1) and allowed us to  
326 genotype the 37 individuals from field populations (Table 4). We found two restriction profiles  
327 for the susceptible genotypes: at 195 and 218 bp sizes. This variation corresponds to a  
328 modification in the intron following the kdr mutation locus leading to the elimination of the  
329 first TasI cut locus. Furthermore, some size variation was observed on total PCR products  
330 probably due to changes in intron sequences. The kdr mutation was found in 5 populations. The  
331 only population without the kdr mutation was from Montardon, the non-treated field.

332

333

### Discussion

334 In the present study, we confirmed the suspected resistance of *O. nubilalis* to lambda-  
335 cyhalothrin and deltamethrin in central France. Results further indicate that both high activity  
336 of detoxification enzymes and the presence of the kdr mutation may be responsible for the  
337 resistance. Results allow a better understanding of the recent problems of control of *O. nubilalis*  
338 with pyrethroids observed in cornfields of central/northern France.

339 Cross-resistance, i.e. that two or more resistances are determined by a similar mechanism, is  
340 often found between active compounds of the pyrethroid family regardless of the mechanism  
341 involved (Zimmer and Nauen 2011, Dykes et al. 2015). In our study, resistance to deltamethrin  
342 was not as high as for lambda-cyhalothrin (Table 3) (highest RR=7.67 as compared to  
343 RR=63.79 respectively) but was strongly correlated with it which is an indication of cross-  
344 resistance. A single population, Epieds en Beauce, did not follow this pattern (high resistance  
345 to lambda-cyhalothrin and little resistance to deltamethrin), calling for a further investigation  
346 of resistance mechanisms.

347 In our sample, both augmented enzymatic activities involved in the detoxification of  
348 insecticides, and the kdr mutation may be responsible for the observed resistance.

349 Analyses of enzymatic activities gave mixed results. First EST activities of resistant individuals  
350 from all populations did not differ from that of the reference strain, suggesting no link to  
351 resistance in contrast to reports in other insect species (Kranthi et al. 2001, Thalavaisundaram  
352 et al. 2012) . Contrarily male resistant individuals exhibited higher MFO activity than the  
353 reference strain in four populations and resistant females from one population exhibited higher  
354 GST activity than the reference. Interestingly, the population where females exhibited higher  
355 GST activity was also the one exhibiting low MFO activity for males, a possible indication that  
356 there could be two profiles of populations where selection would favour one or the other  
357 mechanism because overexpression of the two enzyme families would be too costly.

358 The role of MFO enzymes in resistance to pyrethroids has been observed in other species (Scott  
359 1999, Zimmer and Nauen 2011, Gao et al. 2012) and differential expression of MFOs between  
360 sexes is frequent (Højland et al. 2014). However, differential expression was seldom related to  
361 differences in insecticide resistance (see e.g. female-linked resistance to spinosad in the  
362 housefly, Markussen and Kristensen (2012).. Here these amplified MFO activities were  
363 observed only in males. The females even had lower MFO activity than the reference strain in  
364 two populations. The reference strain had comparable activity to the untreated population  
365 (Montardon), suggesting that it is a satisfactory control. MFO activity was not the only  
366 mechanism determining resistance in these populations. But evidence prove its importance: the  
367 proportion of females was higher in the two ones with little MFO activities than in the four  
368 populations with high MFO activities in males, ( $\chi^2= 4.9$ ,  $df = 1$ ,  $p = 0.026$ ). As for the MFO,  
369 GST have been previously described as responsible in case of Lepidoptera resistance to  
370 pyrethroids (Yu and Nguyen 1992, Bouvier et al. 2002).

371 As suspected for resistance to pyrethroids, a mutation in the sodium channel gene was found in  
372 a resistant individual at the locus of the widespread kdr mutation. This result is in line with the  
373 bioassay results showing cross-resistance between lambda-cyhalothrin and deltamethrin



374 because these two active ingredients target both the sodium channel. Contrarily, we did not find  
375 the super kdr resistance which also confers resistance to pyrethroids in many insects species  
376 (Soderlund and Knipple 2003). However, we investigated super kdr in only one individual so  
377 that we may have missed it. The finding of the kdr mutation allowed developing a molecular  
378 detection tool based on QM13 PCR-RFLP. It enables testing the presence of kdr in 37  
379 individuals at low cost. This tool may provide a large-scale picture of the distribution of this  
380 mutation in our study region. It gave very obvious results and thus appeared as a good candidate  
381 for high throughput monitoring studies that would be very useful for detecting the presence of  
382 the mutation when resistance is suspected or for resistance management programs in situations  
383 where the resistance is caused by kdr. Such a tool has already been developed in many other  
384 pests especially on codling moth (*Cydia pomonella*) or the green peach aphid (*Myzus persicae*)  
385 (Brun-Barale et al. 2005, Cassanelli et al. 2005).PCR-RFLP are routinely used by monitoring  
386 frameworks (IRAC or governmental) to visualise resistance progression in these species. The  
387 tool we developed should be combined with other monitoring techniques when the other  
388 resistance mechanisms will have been deeper investigated.

389 Knock-down resistance mutations are usually considered to be recessive (Soderlund and  
390 Knipple 2003). Here, out of the 26 individuals that survived high insecticide concentration, only  
391 10 were homozygote resistant. Fourteen individuals carried only one copy of the mutated allele  
392 indicating either that its expression was dominant in the conditions of the test or more likely  
393 that these individuals also expressed another resistance mechanism. Lastly, two other  
394 individuals from Semerville were homozygotes for the non-mutated allele at the kdr locus  
395 although they survived high insecticide concentrations. In that case, another mechanism than  
396 the kdr mutation is necessarily at play. Both enzymatic activity and possibly the presence of  
397 another mutation in sodium channel may be involved. Combinations of different resistance  
398 mechanisms including kdr and different MFO genes or transcription factors were found in four

399 house fly strains resistant to the pyrethroid permethrin (Scott and Kasai 2004). More intensive  
400 sampling would be needed to look deeper at the combination of these mechanisms at the  
401 individual level in a panel of resistant populations.

402 The *kdr* mutation was found in locations within 480 km radius. It is therefore disseminated on  
403 the French territory. Such distribution suggests either that the mutation event took place a long  
404 time ago and was spread after that, or that it took place in different locations independently.  
405 The first hypothesis is possible because pyrethroids are used for several decades. Indeed, *O.*  
406 *nubilalis* is not known to have a high mobility (Showers et al. 2001). Furthermore, population  
407 Rouffach from east France, which also carries the mutation is unlikely to exchange genes with  
408 the others as it is a strict monovoltine race showing genetic isolation (Thomas et al. 2003).  
409 Multiple appearances of the *kdr* mutation in different locations was reported in other pest  
410 species (Franck et al. 2012, Rinkevich et al. 2012b, Rinkevich et al. 2012a). Its maintenance in  
411 populations may be related to a small fitness cost as previously observed (Foster et al. 1999,  
412 Sakyi et al. 2005, Brito et al. 2013).

413 The surprisingly long interval between first pyrethroid use and the report of field resistance  
414 could be due to an overall weak selection pressure. The treated area is relatively small in France,  
415 with only one hectare out of six treated due to low levels of damage, and treatment frequency  
416 is limited to 1 or 2 applications per year in France. Moreover, *Ostrinia nubilalis* is polyphagous,  
417 more than 224 plant species have been recorded as hosts by Hodgson in 1928. So it can live in  
418 untreated reguges area, forming pool of susceptibles individuals. The landscape of mixed  
419 farming allow movement of this pest between refuges area and corn crops. This has resulted in  
420 diluting the resistance, making more durable chemical control even monospecific with  
421 pyrethroids. It could also be caused by a recessive inheritance of the mutation under field  
422 conditions making it necessary that the *kdr* allele reaches a high frequency before resistant  
423 (homozygous) individuals are observed. The use of pyrethroids dating back to the 1970's and

424 a local insecticide intensity higher than average in a local region in central France, may explain  
425 why resistance was first reported there. Also, the presence of resistant homozygotes in the tested  
426 individuals suggests that the *kdr* allele is present at high frequency in these populations.

427

428

### **Conclusion**

429 The resistance of *O. nubilalis* to pyrethroids in France seems to result from a combination of  
430 resistance mechanisms. First studies on insecticide resistance reported simple inheritance  
431 (Roush and McKenzie 1987) and semi-dominant expression of resistance (Holloway 1986). But  
432 in contrast, recent research suggests high evolutionary plasticity of resistance (Gao et al. 2012)  
433 and new genomics studies demonstrate the wide variety of genes involved in resistance (Faucon  
434 et al. 2015). This change is probably due to the lasting and widespread pesticide use allowing  
435 the accumulation of multiple mechanisms providing partial resistance to a broad spectrum of  
436 molecules. Understanding how these mechanisms are combined within populations or  
437 individuals is an essential subject for future research.

438

439

440 **Tables**

441 **Table 1:** List of primers used to amplify part of sodium channel gene containing the kdr and  
 442 super-kdr locus in *Ostrinia nubilalis*.

Primer name	Sequence
Skdr-F	5'-GGCCGACACTTAATTTACTCATC-3'
SKdr-R	5'-TTCCCGAAAAGTTGCATACC-3'
Universal M13	5'-GTTGTAAAACGACGGCCAGT-3'
Os-Kdr1	5'-GCACAGTTTCATGATTGTGTTC-3'
Os-Kdr2	5'- CAGGGCCAAGAAAAGGTTAAG -3'
OsNa-R2	5'- GAAGATCCGAAATTTGACAG -3'

443

444

445 **Table 2:** Probit analyses of bioassays performed on seven field populations. Lethal concentrations statistically different from the reference strain  
 446 (non-overlapping confidence intervals) are marked with a star. <sup>1</sup> RR<sup>a</sup> = Resistance Ratio compared to the reference strain. RR<sup>a</sup> = Resistance Ratio  
 447 compared to the most susceptible field population : Montardon for lambda-cyhalothrin, and Rouffach for deltamethrin.

	lambda-cyhalothrin						deltamethrin					
	n	LC50 (CI)	LC90 (CI)	RR <sup>a</sup> (at LC50)	RR <sup>b</sup> (at LC50)	$\chi^2$	n	LC50 (CI)	LC90 (CI)	RR <sup>a</sup> (at LC50)	RR <sup>b</sup> (at LC50)	$\chi^2$
Epieds-en-Beauce, 45	566	137.79 * (69.47 - 178.24)	372.40 * (299.97 - 621.23)	63.79	31.31	11.4	268	12.61 * (8.97 - 16.81)	99.11 * (61.45 - 223.27)	2.22	2.53	28.3
Semerville, 41	484	61.69 * (48.93 - 75.64)	235.30 * (171.08 - 398.44)	28.56	14.02	36.8	328	43.50 * (30.96 - 57.04)	232.24 * (146.70 - 586.37)	7.67	8.73	21.8
Tripleville, 41	443	61.36 * (39.50 - 96.64)	562.99 * (275.80 - 2419.0)	28.41	13.95	17.7	208	39.22 * (28.12 - 62.80)	133.36 * (75.93 - 1118.40)	6.92	7.87	9.9
Labosse, 41	387	27.51 * (10.67 - 230.88)	88.36 * (42.34 - 2.67 10 <sup>8</sup> )	12.74	6.25	7.4	198	11.05 (5.35 - 20.44)	29.04 (17.01 - 388.55)	1.95	2.21	12.8
Binas, 41	257	17.27 * (11.60 - 23.88)	121.08 * (70.54 - 357.26)	8	3.92	20.2	215	20.40 (1.67 - 54.26)	363.50 * (105.32 - 7.25 e+05)	3.6	4.09	5.1
Rouffach, 68	889	5.30 (2.71 - 7.55)	22.16 * (14.46 - 61.25)	2.45	1.20	10.6	438	4.98 (3.44 - 6.46)	12.00 (8.90 - 21.73)	0.88	-	22.3
Montardon, 64	704	4.40 (3.25 - 5.78)	23.05 * (15.24 - 45.91)	2.04	-	40.1	311	5.14 (2.11 - 18.89)	34.28 (12.06 - 284.49)	0.90	1.03	4.4
Ref strain	241	2.16 (1.64 - 2.74)	6.56 (4.84 - 10.85)	-	0.49	14.7	192	5.67 (4.47 - 6.91)	16.12 (12.45 - 24.55)	-	1.10	27.4

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451

452 **Table 3:** Enzymatic activities of EST, GST and MFO on adult abdomens of resistant individuals from seven field populations and a reference  
 453 strain of *O. nubilalis*. Activity ratio is calculated compared to the reference strain. Populations are ranked in descending order according to their  
 454 resistance ratio to lambda-cyhalothrin. n= number of samples. Letters indicate homogenous subsets output obtain with a post hoc test ( $\alpha < 0.05$ ).

455

Population	EST activities (nmol $\alpha$ -naphthol/min/mg prot)		GST activities ( $\mu$ mol of CDNB-GS/min/mg prot)				MFO activities (pg of 7-HC/min/mg prot)			
	mean of EST activity $\pm$ SD	Activity Ratio	mean of GST activity $\pm$ SD		Activity Ratio		mean of MFO activity $\pm$ SD		Activity Ratio	
			Female	Male	Female	Male	Female	Male	Female	Male
<b><i>Epieds-en-Beauce, 45</i></b>	87.83 $\pm$ 50.15 (n=34)	2.16	730.63 $\pm$ 174.58 <sup>ab</sup> (n=17)	1360.77 $\pm$ 591.23 (n=15)	3.24	1.44	86.87 $\pm$ 35.59 <sup>b</sup> (n=20)	599.85 $\pm$ 370.28 <sup>b</sup> (n=15)	2.13	3.67
<b><i>Semerville, 41</i></b>	85.70 $\pm$ 61.89 (n=36)	2.10	1009.58 $\pm$ 209.84 <sup>b</sup> (n=22)	2050.14 $\pm$ 1046.30 (n=16)	4.48	2.17	21.75 $\pm$ 8.68 <sup>a</sup> (n=34)	72.81 $\pm$ 45.73 <sup>a</sup> (n=27)	0.53	0.44
<b><i>Tripleville, 41</i></b>	83.93 $\pm$ 48.22 (n=39)	2.06	887.11 $\pm$ 726.05 <sup>ab</sup> (n=21)	1735.04 $\pm$ 958.35 (n=23)	3.94	1.83	51.71 $\pm$ 18.05 <sup>b</sup> (n=25)	938.35 $\pm$ 493.61 <sup>b</sup> (n=33)	1.27	5.73
<b><i>Labosse, 41</i></b>	27.29 $\pm$ 13.39 (n=10)	0.67	1172.64 $\pm$ 468.35 <sup>ab</sup> (n=9)	492.62 (n=1)	5.21	0.52	19.48 $\pm$ 7.70 <sup>a</sup> (n=20)	161.94 $\pm$ 111.05 <sup>a</sup> (n=7)	0.48	0.99
<b><i>Binas, 41</i></b>	72.37 $\pm$ 46.29 (n=16)	1.78	1002.14 $\pm$ 691.92 <sup>ab</sup> (n=9)	1658.17 $\pm$ 958.14 (n=7)	4.45	1.75	110.20 $\pm$ 25.48 <sup>b</sup> (n=10)	488.04 $\pm$ 221.32 <sup>b</sup> (n=12)	2.71	2.98
<b><i>Rouffach, 68</i></b>	-	-	-	-	-	-	79.47 $\pm$ 74.97 <sup>ab</sup> (n=9)	452.04 $\pm$ 280.11 <sup>b</sup> (n=3)	1.95	2.76
<b><i>Montardon, 64</i></b>	77.17 $\pm$ 26.25 (n=10)	1.89	157.86 $\pm$ 45.67 <sup>a</sup> (n=8)	1011.53 $\pm$ 266.76 (n=2)	0.64	1.07	37.19 $\pm$ 8.66 <sup>b</sup> (n=7)	299.30 $\pm$ 196.62 <sup>ab</sup> (n=4)	0.91	1.83
<b><i>Reference strain</i></b>	40.74 $\pm$ 18.75 (n=93)	-	244.86 $\pm$ 34.87 <sup>a</sup> (n=49)	944.81 $\pm$ 161.46 (n=45)	-	-	40.73 $\pm$ 11.32 <sup>b</sup> (n=50)	163.63 $\pm$ 63.33 <sup>a</sup> (n=29)	-	-

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457

458 **Table 4:** Genotypes of 37 individuals selected (see mat and met) from 6 field populations at

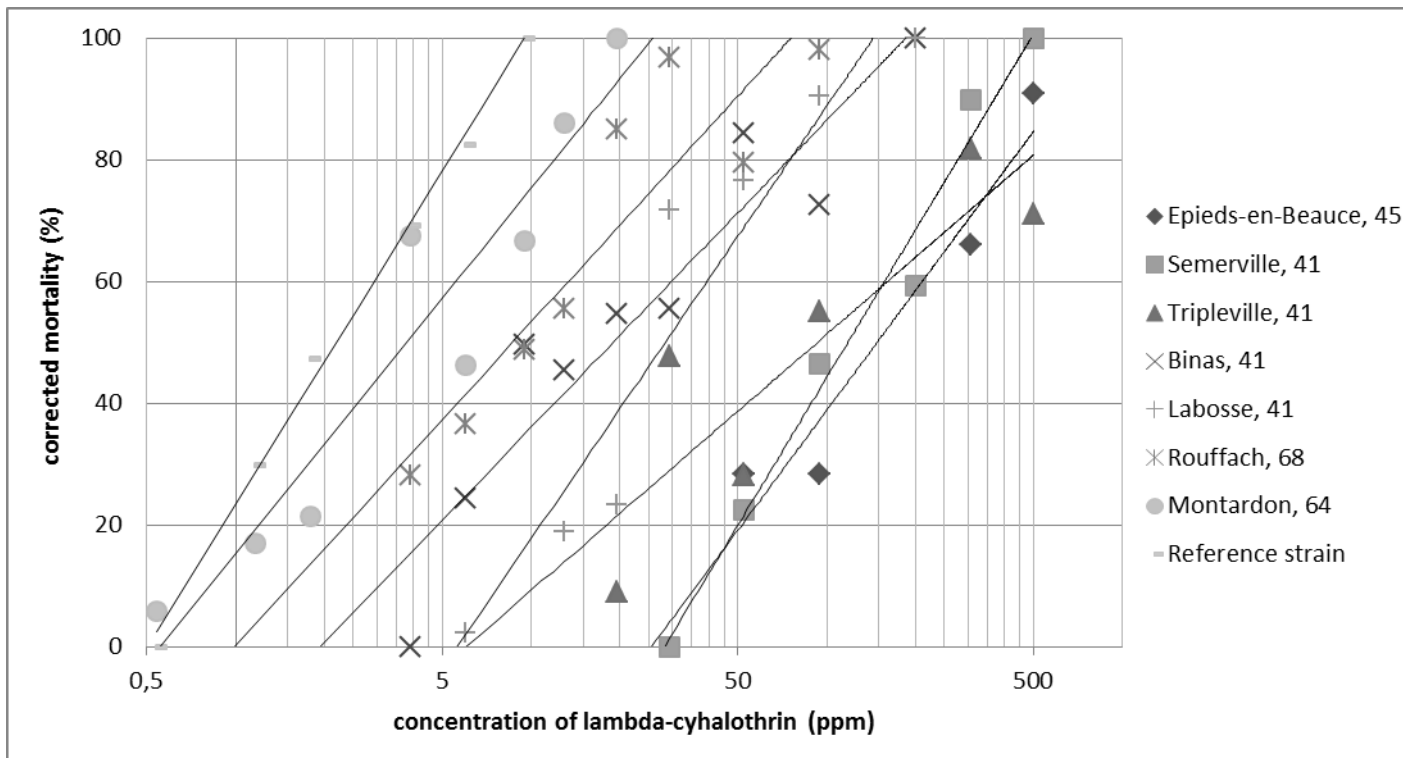
459 the *kdr* locus. The last column gives the resistance ratio to lambda-cyhalothrin at the LC50.

	R-R	R-S	S-S	Res Ratio
Epieds-en-Beauce	1	3		63.79
Semerville	3	8	2	28.56
Tripleville	5	2		28.41
Binas	1			8.00
Rouffach	1	4	2	2.45
Montardon			5	2.04

460

461

462 Figure 1



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473 **Figure legends**

474 **Figure 1:** Concentration-mortality response curves for the bioassay performed with lambda-  
475 cyhalothrin on seven populations and a reference strain of *O. nubilalis*.

476

477 **Figure 2:** (A) Diagram of the domain II of sodium channel protein and proposed folding of  
478 the membrane segments (S1 to S6). (B) Nucleotide and amino acid sequences of the IIS4–  
479 IIS6 region of the susceptible moth (individual n°3 from Montardon) and the resistant one  
480 (individual n°205 from Semerville). The kdr substitution L1014F is placed, primer pair used  
481 for RFLP-PCR is located in the sequence and loci cut by TasI endonuclease are marked by  
482 crosses.

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