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

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Abstract

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

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

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

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

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

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

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

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

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

Materials and Methods

Insect sampling

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

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

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

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

Insecticides

The commercial formulations of lambda-cyhalothrin (Karate Zeon) and deltamethrin (Decis Protech) were purchased from Syngenta (France) and Bayer (France). The insecticides were diluted in osmosed water. Insecticide ranges used to test the reference strain were 0.54 ; 1.17 ; 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 and 30 ppm for deltamethrin. These concentrations were adapted for field populations (see below).

Bioassays

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

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

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

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

Enzymatic activities

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

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

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

Mixed-function oxidase (MFO)

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

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

Glutathione S-transferase (GST)

For protein extraction, anterior half abdomens were crushed in 110 µl of Hepes buffer (50 mM, pH 7) on the ice and the obtained homogenates were centrifuged at 15 000 g for 15 min at 4 °C. Supernatants were stored at -80 °C before use. When all extractions were finished, the supernatants were used as enzyme sources for reactions in a single test to limit handling errors (Bouvier et al. 2002). GST activity was determined in transparent microplates (96-wells, Sterilin®, Newport, UK) using 2.4-dinitro-chlorobenzene (CDNB) as substrate (Nauen and Stumpf 2002). The reaction mixture in one well consisted of 2 µl of enzymatic extract, 198 µl of a solution containing: 10 µl of 50 mM glutathione (GSH), 185 µl of Hepes buffer (50 mM, pH 7.0) and 3 µL of 50 mM CDNB. Three wells per microplate were filled with 2 µl of Hepes buffer (50 mM, pH 7.0) instead of enzyme extract as blank. Absorbance was measured after 2 min of incubation at 25 °C in kinetic mode every 30 seconds at 340nm. Since the CDNB-glutathione adduct was not commercially available, we were unable to build a standard curve, so we used the molar extinction coefficient (9.6 $mM^{-1}.cm^{-1}$) of CDNB-glutathione to convert

absorbance in µmol of CDNB-glutathione. The final specific activity was expressed in µmol of CDNB-glutathione/min/mg of total protein extracted.

Carboxylesterases (EST)

The same protein extracts were used as for GST. Total non-specific EST activity was measured with α-naphthyl acetate (α-NA) as substrate (Reyes 2007). The reaction mixture was 1 µl of protein extract and 194 µl of 30 µM α-NA in Hepes buffer (50 mM, pH 7.0) in each microplate well. After 20 min of incubation at 22 °C in darkness, the reaction was stopped and coloured by adding 55 µl of 0.2 % Fast Garnet GBC diluted in 2.5 % sodium dodecyl sulphate solution. Absorbance was recorded at 590 nm, after incubation for 20 min in darkness at room temperature.

The standard curve with α-Naphtol (0-18 nmoles/well) was elaborated to express activity in nmoles of product/min/mg of total proteins.

Sequencing and detecting the *kdr* mutation

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

Sequencing the kdr mutation

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

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

PCR amplifications were performed with a Mastercycler thermocycler (Eppendorf). Each reaction had a volume of 25 µl containing the following MIX : 1X GoTaq® Flexi Buffer, 1.5 mM $MgCl_2$, and 0.1 mg/ml Bovine Serum Albumin, 200 µM of each dNTPs, 0.4 µM of each Forward and Reverse primers, 1 unit of GoTaq® Flexi DNA Polymerase (Promega) and 4 µl of DNA template. The PCR conditions were: 3 min at 95 °C followed by 35 cycles at 95 °C for 45s, 50 °C for 1 min, and 72 °C of elongation for 1.5 min with a final extension step at 72 °C for 10 min.

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

Detection of the kdr mutation

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

GTTGTAAAACGACGGCCAGT-3' M13-Tail; at its 5' end, the same universal 6-FAM (6-carboxyfluorescein) labelled M13-Tail was used for fluorescence detection. We used a ratio of 1 M13-tailed-Os-Kdr1 primer for 10 Labelled universal M13-tail and 10 Os-Kdr2 reverse primers. We found two size of amplicon, probably due to intron variability: 268 and 273 bp. PCR amplifications were carried out with a Mastercycler thermocycler (Eppendorf). The reaction occurred in 12 µl containing : 1X GoTaq® Flexi Buffer, 1.5 mM $MgCl_2$, 0.1 mg/ml Bovine Serum Albumin, 200 µM of each dNTPs, 0.4 µM of each labelled M13-Tail and specific reverse primer, 0.04 µM of M13-Tailed specific forward primer, 1 unit of GoTaq® Flexi DNA Polymerase (Promega) and 2 µl of DNA template. The PCR conditions were: 3 min at 95 °C 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 for 30s, 50 °C for 45 s, 72 °C for 45s with a final extension step at 72 °C for 20 min.

All the PCR products were digested at 65 °C for 16 hours with two units of Tas I endonuclease and 1X of Buffer N°B (Fermentas) in 20 µl of final reaction volume. Ten µl of digested products were diluted in 100 µl H2O; 2 µl of this dilution, 7.8 µl of HiDi formamide, and 0.2 µl GeneScan™- 600 LIZ® Size standard (Applied Biosystems) were injected on an ABI 3730xl DNA Analyzer (Applied Biosystems) using POP7 polymer. These genotyping runs were analysed using GeneMapper® V4.1 Analysis Software (Applied Biosystems).

Each revealed fragment length was defined as an allele.

Statistical analyses

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

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

Results

Toxicological tests

There was a wide diversity of susceptibility to lambda-cyhalothrin in the field as shown by the continuous range of concentration-mortality response curves in toxicological tests (Figure 1). All field populations showed a lower susceptibility to lambda-cyhalothrin than the reference strain. Resistance ratios at LC50 varied from 2.04 to 63.79 for the less (Montardon) to the most resistant population (Epieds-en-Beauce) (Table 2).

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

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

Enzymatic activities

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

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

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

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

Genetic analyses

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

have not found the super kdr mutation in the resistant individual sequenced.

The PCR-RFLP detection tool gave clear results (Supplementary Figure 1) and allowed us to genotype the 37 individuals from field populations (Table 4). We found two restriction profiles for the susceptible genotypes: at 195 and 218 bp sizes. This variation corresponds to a modification in the intron following the kdr mutation locus leading to the elimination of the first TasI cut locus. Furthermore, some size variation was observed on total PCR products probably due to changes in intron sequences. The kdr mutation was found in 5 populations. The only population without the kdr mutation was from Montardon, the non-treated field.

Discussion

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

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

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

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

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

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

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

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

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

Conclusion

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

Tables

Table 1: List of primers used to amplify part of sodium channel gene containing the kdr and 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'

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

	lambda-cyhalothrin						deltamethrin					
	n	**LC50 (CI)**	**LC90 (CI)**	**RR^a (at LC50)**	**RR^b (at LC50)**	**χ^2**	**n**	**LC50 (CI)**	**LC90 (CI)**	**RR^a (at LC50)**	**RR^b (at LC50)**	**χ^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 10^8)	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

450

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

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

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

Figure 1

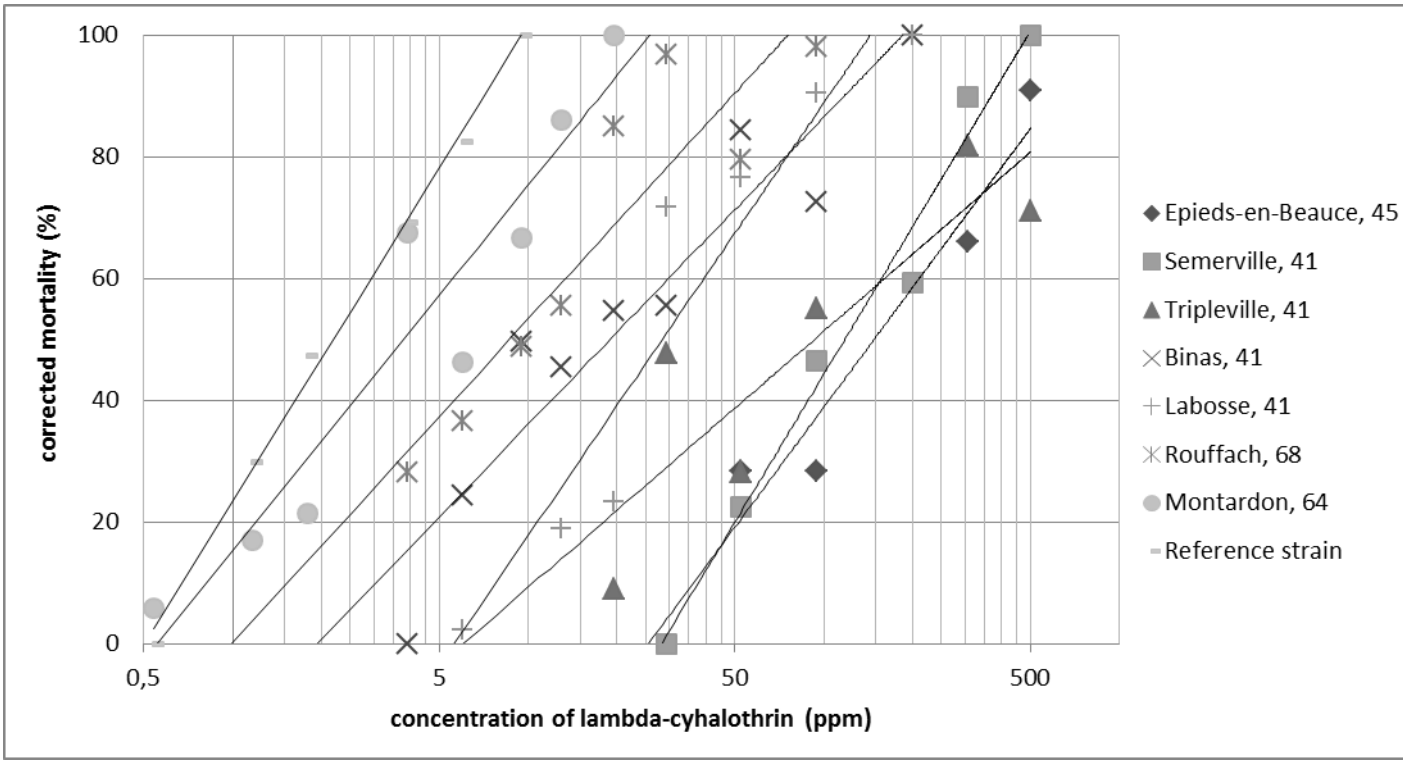

Figure 2

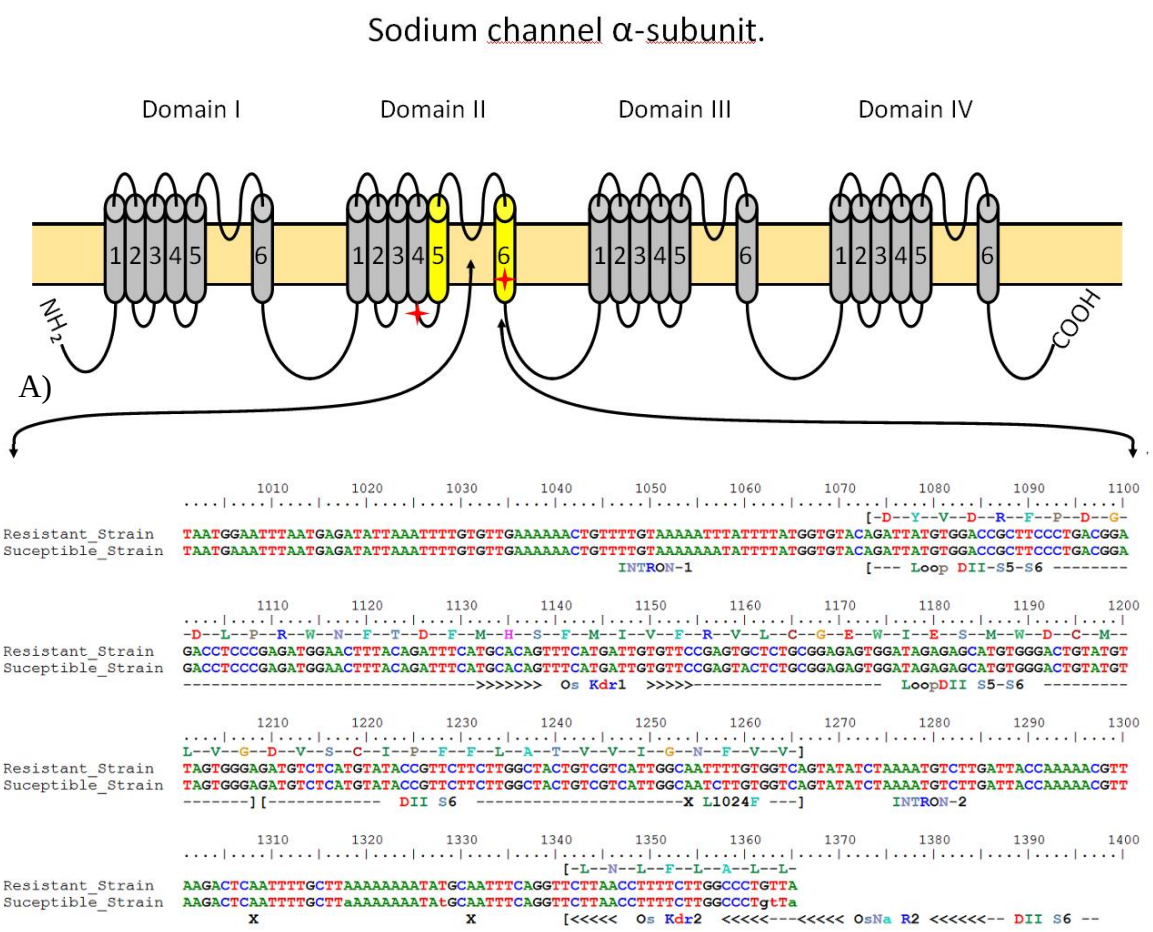
Sodium channel α-subunit.
Domain I
Domain II
Domain III
Domain IV
1 2 3 4 5 6
NH2
COOH
A)
Resistant_Strain
Suceptible_Strain
INTRON-1
[--- Loop DII-S5-S6 --------
Os Kdr1
LoopDII S5-S6
DII S6
X L1024F ---]
INTRON-2
Os Kdr2
OsNa R2
DII S6 --
B)

Figure legends

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

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

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