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Tools for resistance monitoring in the oriental fruit moth (Lepidoptera: Tortricidae) and first assessment in Brazilian populations

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Abstract

In southern Brazilian apple orchards, predominantly organophosphates are used to control the oriental fruit moth, *Cydia molesta* Busck, but control failures often occur. Therefore the susceptibility of three *C. molesta* Brazilian populations was investigated to five insecticides of different groups and modes of action, in comparison with a susceptible laboratory strain mass reared in Southern France for over ten years. At the same time, comparative biochemical and genetic analysis were performed, assessing the activities of the detoxification enzymatic systems and sequencing a gene of insecticide molecular target to find out markers associated with resistance. The three Brazilian populations were significantly resistant to chlorpyrifos ethyl compared to the reference strain. One of the field populations, which had been frequently exposed to deltamethrin treatments, showed significant decreasing susceptibility to this compound, whereas none of the three populations had loss of susceptibility to tebufenozide and thiacloprid when compared to the reference strain. All three populations had slight but significant increases of Glutathione-S-transferase and carboxylesterases activities, and significant decrease of specific acetyl cholinesterase (AChE) activities compared to the reference. Only the most resistant population to chlorpyrifos exhibited a significantly higher mixed-function oxidase activity than the reference. The acetyl cholinesterase of females was significantly less inhibited by carbaryl in the Brazilian populations than in the reference strain (1.7- to 2.5-fold), and this difference was not expressed in the male moth. However, no mutation in the MACE locus was detected. These biological and molecular characterisations of adaptive response to insecticides in *C. molesta* provide tools for early detection of insecticide resistance in field populations of this pest.

Key words: *Cydia molesta*, insecticide resistance, monitoring, mechanism, target mutation

Abstract in Portuguese

Os inseticidas organofosforados são utilizados predominantemente para controle da mariposa oriental, *Cydia molesta* Busck em pomares de maçã na região Sul brasileira, mas ocorrem frequentemente perda de eficiência de controle. Portanto, a suscetibilidade de três populações brasileiras de *C. molesta* foi estudada a cinco inseticidas de diferentes grupos e modos de ação, comparando com população suscetível de laboratório criadas no sul da França há mais de dez anos. Ao mesmo tempo, foram realizados testes comparativos bioquímicos e análises genéticas, avaliando as atividades dos sistemas de desintoxicação enzimática e sequenciamento de um gene, procurando encontrar marcadores associados à resistência. As três populações brasileiras foram significativamente resistente ao clorpirifós etil em comparação com a população de referência. Uma das populações de campo, que tinha sido frequentemente expostos a deltametrina, apresentaram diminuição significativa de sensibilidade a este composto, ao passo que nenhum dos três populações apresentaram uma perda de susceptibilidade para tebufenozide e tiaclopride, quando comparada com a população de referência. As três populações apresentaram aumentos significativos de glutathione-S-transferase e atividades carboxilesterases e diminuíram a atividade de acetilcolinesterase (AChE) em relação à referência. Apenas a população mais resistente ao clorpirifós apresentou significativa atividade de oxidase de função mista em relação a referência. A acetilcolinesterase de fêmeas foi significativamente menos inibida por carbaryl na população brasileira do que na população de referência (1,7 a 2,5 vezes), e essa diferença não foi expressa em machos. No entanto, nenhuma mutação no locus MACE foi detectado. Estas caracterizações biológicas e moleculares, da resposta adaptativa de *C. molesta* aos inseticidas, fornece ferramentas para a detecção precoce da resistência em populações de campo.

Palavras chave: *Cydia molesta*, resistência a inseticida, monitoramento, mecanismo, mutação

The oriental fruit moth, *Cydia molesta* (Busk), is a damaging lepidopteran pest of peach tree (*Prunus persicae* (Linné)) orchards. *C. molesta* larvae attack both shoots and fruits of its native host plant, but this moth is also known to feed on quince (*Cydonia oblonga* (Mill.)), apricot (*Prunus armeniaca* (Linné)), almond (*Prunus dulcis* (Mill.)), medlar (*Mespilus germanica* (Bosc.)), and to a minor degree on cherry (*Prunus cerasus* (Linné)) and plum (*Prunus domestica* (Linné)) (Balachowsky 1966). Moreover, while this species was previously expected to migrate towards pome fruits in the late generations after the harvest of stone fruits, *C. molesta* has acquired the ability to complete its life cycle on American and European pome fruit orchards and is causing heavy damages on apple and pears (Reis et al. 1988, Usmani and Shearer 2001, Natale et al. 2003).

In South Brazil *C. molesta* is present since the early 1980s (Lorenzato 1988), and its damages were first recorded in Vacaria (Rio Grande do Sul) and Fraiburgo (Santa Catarina) then during the 90's in São Joaquim (Santa Catarina) and Porto Amazonas (Paraná). The control of *C. molesta* in Brazil involved exclusively neurotoxic insecticides including numerous organophosphates until the end of 1990s. Tebufenozide and novaluron are the only insect growth regulators registered against *C. molesta* in Brazil (Andrei 2009). These insecticides are aimed to control simultaneously *C. molesta* and secondary pests, including *Anastrepha fraterculus* (Wiedemann) (Diptera: Tethritidae) and *Bonagota cranaodes* (Meyrick) (Lepidoptera: Tortricidae) (Botton et al. 2000).

In Brazilian apple orchards and in South-European peach tree orchards as well, the protection against *C. molesta* requires up to ten treatments per year (Monteiro et al. 2009, Siegwart 2010). Despite this high insecticide pressure, control failures were recorded in several locations of both production areas during the last two years (Monteiro and Souza 2010, Monteiro et al. 2009,

84 Siegwart 2010b). This could be the result of the selection of insecticide resistance, as it already
 85 occurred for organophosphates and carbamates in North American populations of *C. molesta*
 86 (Kanga et al. 1997, Pree et al. 1998).

87 Insecticide resistance management requires a discontinuous selection process, which may be
 88 obtained from the use of non-chemical control methods (Roush and Tabashnik 1990, Monteiro et
 89 al. 2008) or by alternating insecticide compounds with different modes of action. The
 90 implementation of new compounds is thus of interest to counter the selection of resistance to
 91 neurotoxic compounds that may result from their intensive and often exclusive use in Europe
 92 (mainly pyrethroids) or in South America (mainly organophosphates). New neocotinoid and insect
 93 growth regulator families could be interesting tools, due to the fact that several neurotoxic active
 94 ingredients are withdrawn from European and South American registration.

95 Early detection of insecticide resistance is also needed to avoid the rapid spread of this
 96 phenomenon and to allow the implementation of resistance management strategies (Kanga et al.
 97 2003). Studies on the North American population showed an increase of Esterase in resistant
 98 populations compared to the susceptible ones, along with a decreased inhibition of acetylcholine
 99 esterase by carbamates and organophosphates (Kanga et al. 2003), indicating a probable
 100 mutation in this enzyme gene.

101 In this study, the resistance status of Brazilian populations of *C. molesta* collected in more or less
 102 intensively sprayed orchards was investigated to five active ingredients from different insecticide
 103 families: (i) neonicotinoid, (ii) pyrethroid, (iii) moulting activating compound (MAC), (iv)
 104 carbamate and (v) organophosphate (OP). Bioassays were paired with metabolic analysis and
 105 gene sequencing of insecticide molecular targets, to identify markers associated with the
 106 resistance to these different insecticide groups. Enzyme assays were designed to assess the

activity of three systems involved in insecticides detoxication in numerous insect pests: Glutathione S-transferases (GST), mixed function oxydases (MFO) and carboxylesterases (EST) (Yasutomi 1983, Oppenoorph 1985). The total acetylcholine esterase activity which may vary resulting in resistance to organophosphates or carbamates (Zhu and Gao 1999) was estimated. Finally, genes coding for the two forms of acetylcholine esterase (AChE) were partially sequenced. The whole methodology was designed to define molecular and multi-metabolic diagnostic tools to investigate the combination of mechanisms involved in resistance phenomenon.

Material and methods

Insects

Three Brazilian populations of *C. molesta* were sampled in apple orchards in Vacaria (Rio Grande do Sul state), Lages (Santa Catarina state), and Porto Amazonas (Paraná state). A susceptible laboratory strain (S_{it}) originating from Italian peach tree orchards (Provided by Fabio Molinari, University of Piacenza, Italy) was used as reference. The Vacaria population was collected in 2005 and reared in the laboratory on artificial diet (Guennelon et al. 1981) during 3 years with two further introductions of new genetic material from the same orchard. This orchard had mainly been protected using pyrethroid and organophosphate applications previous to collecting the population. The other two Brazilian populations were field collected in early 2008 in orchards where failure of chemical protection occurred. In 2006/2007 season five OPs were applied in Porto orchard while Lages received seven organophosphates and two MAC. Close to 10% damages were recorded in 2007 in Lages orchard despite this protection program (unpublished data). Porto and Lages populations were reared in Parana Federal University, Curitiba (Brazil) during two generations. The three Brazilian populations were then transferred at

INRA Avignon, where analyses were performed. These analyses were completed during three successive generations, required to multiply the insects and for preliminary tests to set the adequate concentrations of the different insecticide and the methodology of enzyme analysis. The rearing of the field populations and of the reference strain was conducted at $25 \pm 1^\circ\text{C}$, 70% RH under 16 L: 8 D photoperiod. Egg laying was obtained in plastic tubes (8cm in diameter, 20 cm long) closed on both sides with gaze. Eggs were placed on artificial diet (Guennelon et al 1981) in a plastic container (30 x 14 x 10 cm) for larval development. Larvae pupated in gaze strips, in plastic containers. The reference strain S_{it} had been mass reared on artificial diet for 10 years in Crop Protection Service of Lyon (France), without any insecticide exposure.

Insecticides

Susceptibility of the populations to the OP: chlorpyrifos-ethyl (Pyrinex ME, 250 g/l, Makhteshim-Agan France), to the neonicotinoid: thiacloprid (Calypso, 480 g/l, Bayer CropScience France), to the pyrethroid: deltamethrin (Decis, 15 g/l, Bayer CropScience France), to the carbamate: carbaryl (Sevin, 85% wettable powder, Certis France) and to the benzhydrazid: tebufenozide were assessed. Fresh dilutions of formulated insecticides were prepared in distilled water for bioassays on neonates.

Bioassays

Microplate (96-wells, Sterilin®) wells were filled with 150 μL of artificial diet (Stonefly Industries Ltd, Rochester, NY), and 6 μL of each insecticide solution were applied to the diet's surface according to Reyes and Sauphanor (2008). For each population, concentration-response relationships were established per insecticide. Six concentrations giving between 0 and 100% mortality were used. Distilled water replaced insecticide in the control (Fuentes-Contreras et al. 2007). Newly hatched larvae (0–4 h old) were individually placed in the wells. Mortality was

recorded after 7 days at 25°C. A larva was considered as dead when not responding to a probe with dissecting forceps. Missing larvae (a mean of 1.6% in the whole experiment) were subtracted from the initial number.

Enzymatic activities

Glutathione S-transferase (GST), mixed-function oxidase (MFO), esterase (EST) and acetylcholine esterase (AChE) activities were evaluated on adult (head for AChE activity and inhibition, abdomen for MFO and thorax for other enzymes). At least 12 insects per sex and per population were analysed for each enzymatic system. Fluorescence and absorbance were measured using a microplate reader (HTS 7000, Perkin Elmer). In order to obtain activities of the three enzyme systems on each insect, each part of adult body was used as specific enzyme extract.

Enzyme extracts. EST and GST activities were evaluated using single thorax homogenized in 110µl of 50mM Hepes buffer (pH 7). For AChE activity, one head was homogenized on ice in 50µl of 50mM phosphate buffer with 0.5% Triton (pH 7.2). For AChE inhibition five heads of adults were pooled and homogenized in 250 µl of ice-cold phosphate buffer (0.05M; pH 7.2) containing 0.5% Triton. The homogenates obtained from each insect segment were centrifuged at 15 000 × g for 15 min at 4°C, and the supernatants were used as enzyme sources (Bouvier et al. 2002). The protein content of each sample was measured (Bradford 1976) using bovine serum albumin to build the standard curve.

Glutathione S-transferases. GST activity was determined in black microplates (96-wells, Costar®) using monochlorobimane (MCB) as substrate (Nauen and Stumpf 2002). The reaction mixture in one well consisted of 30µl of enzymatic extract, 170µl of a solution containing: 6µl of 100mM glutathione (GSH), 162µl of Hepes buffer (50mM, pH7.0) and 2µL of 30mM MCB.

Wells with Hepes buffer instead of enzyme extract were used as controls. Fluorescence was measured after 20 min incubation at 22°C, with 380 nm excitation and 450 nm emission filters. Since the bimane-glutathione adduct was not commercially available, the activity was expressed as fluorescence units per min per µg of total protein extracted.

Esterases. Total non-specific EST activity was measured with α-naphthyl acetate (α-NA) and para nitrophenyl acetate (p-NA) as substrates (Reyes et al, in press). The reaction mixture for the first substrate was 1µl of protein extract and 194µl of 30µM α-NA in Hepes buffer (50mM, pH 7.0) in each microplate well. The reaction was stopped and coloured after 20 min incubation at 22°C in darkness, by adding 55µl of 0.2% Fast garnet GBC in 2.5% sodium dodecyl sulphate solution. Absorbance was recorded at 590nm, after incubation during 20 min in darkness at room temperature.

The reaction mixture for the second substrate was 2.5µl of protein extract, 2.5µl of p-NA (50 mM in DMSO) in 245µl of Hepes buffer (50 mM, pH 7) with EDTA (1 mM) in each well. Absorbance was recorded at 405nm each minute (kinetic mode) during 10min. If the stabilisation of speed reaction occurred, the difference of OD per min was calculated, otherwise the measurement was repeated. Two standard curves with α-Naphtol (0-18nmoles/well) or p-nitrophenol (0-37.5nmoles/well) were elaborated to express activity in nmoles of product/min/mg of total proteins.

Mixed-function oxidases. The MFO activity was determined using 7-ethoxycoumarin O-deethylation (ECOD) (Ulrich 1972) adapted for in vivo analysis in microplate. Twenty four fresh insects of each strain were analysed. Adult abdomens were dissected and homogenised in incubation solution: 100µl of Hepes buffer (50mM pH 7) with 7-ethoxycoumarin (0.4mM); on ice and centrifuged at 15 000 × g for 2 min at 2°C. Supernatants were individually placed in

wells of black microplates (96-wells, Costar®). After 4h incubation at 30°C, the reaction was stopped by adding 100µL of 1.5M glycine buffer (pH 10.3). The 7-hydroxycoumarin (HC) fluorescence was quantified with 380 nm excitation and 465 nm emission filters. Four wells receiving glycine buffer previous to incubation were used as control. The activity was expressed as pg of 7-HC/insect/min thanks to a standard curve of 7-Hydroxycoumarine (0.5-4.5 nmoles/well).

Acetylcholine esterase. The AChE activity was determined using acetylthiocholine (ASCh) as substrate (Ellman et al. 1961). To analyse numerous samples, Ellman's protocol was adapted for microplate analysis. The enzyme activity was measured by increase of yellow coloration due to the formation of thionitrobenzoate, based on the following reactions:

Nineteen to 40 insect extracts were analysed per sex and population. The reaction mixture in one well was composed of 138.5µl of phosphate buffer (0.1M, pH 8.0), 5µl of DTNB 0.01M (0.3 mM final concentration), 1.5µl ASCh 0.1M (1M final concentration) and 5µl of protein extract. Four wells receiving phosphate buffer (0.1M, pH 8) instead of protein extract were used as control. Absorbance was recorded at 405 nm every minute (kinetic mode) during 10 min. When the reaction speed was stabilized, the activity was calculated and expressed in nmoles of Thiocholine/min/mg of total protein thanks to a standard curves with DTT (0-4.5 nmoles/well) instead of thiocholine (Ellman et al. 1961). As DTT has two functional groups -SH this standard curve has to be divided by two before using.

The inhibition of AChE activity by carbaryl was assessed using 25 insects per sex and population. 20µl of carbaryl dissolved in ethanol was placed in each well of a microplate, and the

solvent was evaporated to dryness before addition of reaction mixture. Preliminary studies were conducted to estimate the representative range of insecticide concentrations. On this prepared plate, 10µl of extracts were incubated at 4°C during 20min. In the control wells the insecticide was replaced by the solvent alone and in the blank by 0.01 M of Eserine. The reaction was initiated by addition of a solution containing 133.5µl of phosphate buffer (0.1M, pH 8), 5µl of DTNB 0.01M (0.3mM final concentration) and 1.5µl ASCh 0.1M (1 M final concentration). The absorbance was recorded every 5 min for a total period of 40 min. Three replicates of seven concentrations were used in each test. Concentration of insecticides causing 50% inhibition (IC50) of AChE activity were estimated though probit analysis (Russell et al. 1977). Difference in inhibition among genotypes were considered not significant if the 95% CL of the inhibition ratio at the IC 50 level of the susceptible strain included 1.0 (Robertson and Preisler 1992).

Sequencing the molecular targets of insecticides

Eight individuals were sequenced for ace1: one from S_{it}, one Porto, two Vacaria and two Lages the last two are a Porto and a Vacaria survival of biotests at 1200ppm of chlorpirifos. Theses last two individual are used to sequence ace2 gene.

We performed the total DNA extraction of *C. molesta* using a hexadecyl-trimethyl-ammonium bromide (CTAB) protocol (Murray and Thompson 1980). Adults were individually ground in 200µl of proteinase K (0.3 mg/ml) and incubated over night at 56°C. Lysis occurred at 65°C during 1h by adding 300µl of lysis buffer (Tris-HCl 200mM, EDTA 50mM, NaCl 2M, CTAB 2%) and 100µl of sarcosyl 5%. Proteins were precipitated and separated by chlorophorm-isoamylalcohol (24:1) treatment. Nucleic acids were precipitated at -20°C after adding one volume of isopropanol. DNA pellet was washed in ethanol and resuspended in 60µl of water. PCR amplifications were carried out in a 25µL reaction volume containing, 1X GoTaq buffer

(Promega), 200µM of each dNTPs, 0.4µM of each primer, one Unit of GoTaq and 2µl of DNA template.

For amplification in the first gene of AChE (ace1), we used primers: ACE 1S (5'-cccagacctgttgaaagctg-3') and ACE 1R (5'-tgctctctggtaatgcctacg-3') and for amplification in the second gene (ace2) we used primers: ACE 2S (5'-gtgccgcagcatttaagagt-3') and ACE 2R (5'-tgccctccttcattcattgtg-3')

Thermal conditions were : 94°C for 3min, followed by 35 cycles of 94°C for 30s, 55°C for 60s and 74°C for 2min. For ace1, primers were designed using a sequence of the related tortricid moth *Cydia Pomonella* L (Lepidoptera: Tortricidae). This sequence name is cydpom-ace1, noted in the GenBank database under the accession number DQ267977 (Cassanelli et al. 2006). For ace2 the cDNA sequence of the gene (accession number: HM775184) was provided by S. Cassanelli (University of Modena and Reggio Emilia, Italie).

The PCR fragments obtained were purified after visualisation using 1% agarose gel with the QIAquick® Gel Extraction Kit and directly sequenced (Genome express, Meylan, France). Data analysis was performed with the BioEdit software (Tom Hall, Carlsbad, US).

Statistical analyses

A probit analysis on corrected mortalities (Abbott 1925) was done to determine the LC50 values (Raymond 1985). Resistance ratios at the LC50 (RR₅₀, which is the ratio between the LC50 of the resistant strain and the LC50 of the susceptible strain) and their 95% CL were calculated. The LC50s were considered different when the 95% CL of their RR₅₀ did not include 1. Biochemical data were subjected to analysis of variance (ANOVA). Means were compared by the protected least significant difference Tukey test (P < 0.05) using the software R (Bell Laboratories, Murray Hill, US).

Results

Laboratory bioassays

The three Brazilian populations tested were significantly less susceptible to chlorpyrifos than the European reference strain S_{it} , with RR_{50} ranging from 2.70 to 2.98 (Table 1). The Vacaria population was also significantly resistant to deltamethrin ($RR_{50}=1.80$) while the two other populations were not. Vacaria population was also the less susceptible to carbaryl, with 1.00 – 2.08 values of the 95% CL of its RR_{50} (Table 1). Surprisingly, two populations were significantly less susceptible to thiacloprid than the reference strain ($RR_{50}=0.61$ and 0.43 for Vacaria and Porto, respectively), and the Lages population was two times less susceptible to tebufenozide than S_{it} ($RR_{50}=0.48$).

Despite differences in their selection pressure, the three Brazilian codling moth populations did not exhibit strong differences in their insecticide susceptibility. All of them responded similarly to chlorpyrifos ethyl. However the Vacaria population was 2-fold less susceptible to deltamethrin than Porto and Lages (Vacaria vs Porto $RR_{50}= 2.04$ (1.32 -3.16); Vacaria vs Lages $RR_{50}=1.96$ (1.27 – 3.05)). In the same way, Porto population was 2.19 (1.60 – 2.99) times more susceptible to thiacloprid than Lages, and Lages was 2.04 (1.53 – 2.72) times more susceptible to tebufenozide than Vacaria .

Enzymatic activities

The three Brazilian populations exhibited significantly higher GST activities than the S_{it} strain ($F = 34.11$, $df = 39$, $P < 0.001$, $F = 25.34$, $df = 43$, $P < 0.001$, $F = 15.95$, $df = 45$, $P < 0.001$ for Lages, Porto and Vacaria, respectively) (Figure 1a). The highest enzymatic ratio was obtained between Lages and S_{it} strain (1.7 fold). No sex-linked variability was observed for this enzyme

system ($F = 2.309$, $df = 1$, $P = 0.133$) (Data not shown). EST activities measured using both α -NA and p-NA substrates followed similar patterns as GST activity (Figure 1b), with significantly higher values in the three Brazilian populations than in the S_{it} strain ($F = 29.060$, $df = 40$, $P < 0.001$; $F = 35.112$, $df = 44$, $P < 0.001$; $F = 14.914$, $df = 46$, $P < 0.001$ with α -NA for Lages, Porto and Vacaria, respectively; $F = 31.400$, $df = 39$, $P < 0.001$; $F = 29.863$, $df = 43$, $P < 0.001$; $F = 17.512$, $df = 43$, $P = 0.001$ with p-NA for Lages, Porto and Vacaria, respectively). The highest enzymatic ratio was between Lages and S_{it} strain: 2.0 fold for α -NA and 1.7 fold for p-NA. The Lages populations, collected in orchards where failure of chemical protection occurred, had higher GST and α -NA activities than the Vacaria population while the Porto population exhibited intermediate activities for both enzyme systems. These three populations did not differ significantly from each other for p-NA EST activities.

A different pattern was observed for specific acetylcholine esterase activity, with a significantly higher activity in the S_{it} strain than in Brazilians populations ($F = 87.4077$, $df = 211$, $P < 0.001$). But as for GST and EST enzyme systems, the Vacaria population exhibited a lower specific AChE activity than Porto and Lages ($F = 50.9626$, $df = 105$, $P < 0.0001$ and $F = 27.4031$, $df = 126$, $P < 0.0001$, respectively) (Figure 1c). No sex-linked variability was observed for AChE activities ($F = 0.375$, $df = 211$, $P = 0.5410$).

Only the Lages population had significantly higher MFO activity than the S_{it} strain ($F = 7.7$, $df = 46$, $P = 0.008$). Moreover, activity ratio is low (1.4-fold), and the three Brazilian populations did not differ significantly from each other for this character. All tested females exhibited a higher MFO activity than males ($F = 11.3$, $df = 84$, $P < 0.002$) (Figure 2), due to their significant bigger size (data not shown). This difference was verified whatever the population (S_{it} : $T = 7.9$, $df = 22$, $P < 0.001$; Porto: $T = 18.2$, $df = 22$, $P < 0.001$; Vacaria: $T = 10.9$, $df = 15$, $P < 0.005$; Lages: $T =$

8.2, $df = 22$, $P < 0.001$). The inhibition of the AchE activity by carbaryl was significantly lower in the Brazilian population than in the S_{it} strain, excepted for the males of the Vacaria population (Table 2). The AChE of Lages population was also less susceptible to the inhibition by carbaryl than that of Vacaria moths.

Gene sequencing of acetylcholinesterase

Parts of the two AChE genes (*ace1* and *ace2*) were sequenced for the first time in *C. molesta* in order to search for a mutation potentially involved in organophosphate resistance. No differences were found between the four oriental fruit moth populations in the 850 pb segments of *ace1* gene that were sequenced (AN in GenBank:HM775185). 74 points differences were found between the DNA sequences of *C. molesta* and *C. pomonella*, three of them being responsible of protein variation : I168V ; P272A and I369T (Numbers correspond to *C. pomonella* (numbering DQ267977)). These three variant amino acids exist in other species. They are presumably not involved in the enzyme conformation and activity. The second gene known to code acetylcholinesterase in *Cydia* genus, *ace2* was partially sequenced in individuals showing high tolerance to chlorpyrifos in our bioassays (AN in GenBank: HM775184). But none sequences differences was found between our S_{it} strain and this individuals.

Discussion

The first aim of this study was to set up in *C. molesta* a method previously developed in *C. pomonella* allowing the early detection of insecticide resistance in field populations (Reyes and Sauphanor 2008). This was achieved through the implementation of bioassays with targeted insecticides, enzymatic diagnostic based on the main systems involved in insecticide metabolism and sequencing of the molecular target of organophosphates.

The microplate bioassay on neonates already described for *C. pomonella* (Reyes and Sauphanor 2008) proved appropriate for *C. molesta* and provided reliable results for the five tested insecticides, highlighting small but significant differences between the tested populations and the reference susceptible strain. The three Brazilian populations exhibited a higher tolerance to chlopyrifos than the reference strain S_{it} , in coherence with the frequent applications of organophosphorous insecticides in Brazilian orchards to prevent *C. molesta* injury. Such reduced susceptibility to organophosphates may partially explain the heavy damages on fruits caused in these orchards by *C. molesta* during the last five years. On the other hand, Vacaria population was significantly more tolerant to deltamethrin than Porto and Lages, probably due to the fact that pyrethroids were more frequently used before the year 2000 than nowadays. Indeed Vacaria is one of the oldest and the largest growing area for apple orchards in Brazil, planted in the beginning of 1980. Lages and Porto orchards were planted more recently, in 1990 and 1998, respectively, and therefore received only few pyrethroid treatments during their production period.

In our study the reliability of the reference strain has to be considered carefully. It was collected in Italy over 15 years ago in an untreated peach tree orchard, and was then maintained in continuous mass rearing in the laboratory without any selection pressure. It is thus expected to be susceptible to insecticides, but also to have a genetic background strongly different from the populations more recently sampled, in apple orchards of a distant continent. All our populations had been reared on artificial diet for a few generations in the same conditions previous to the study, avoiding the differential induction of digestive enzymes by the host plants. However we cannot exclude that the differences of enzyme activities recorded between the field populations

and the reference result from these distant genetic backgrounds rather than from an adaptive response to the insecticide pressure. The response to insecticide and the enzyme activities were often observed to be under dependence on the host plant, as demonstrated for the susceptibility of *Epiphyas postvittana* (Lepidoptera: Tortricidae) to organophosphates (Robertson, et al. 1990). The comparison of the responses to insecticides and of biochemical characteristics between the three Brazilian populations is thus meaningful.

The three Brazilian populations exhibited a reduced susceptibility to chlorpyrifos compared to the reference strain, together with a decreased specific activity of the AChE target of Ops and carbamates. It is noticeable that the Vacaria population owning the lowest specific AChE activity was also the less susceptible to carbaryl. Similar OP resistance associated to reduced AChE's affinity for acetylthiocholine substrate was previously described in *C. pomonella* (Reuveny et al 2004). This resistance to chlorpyrifos in OFM populations was also associated with an increase of GST and EST (for both a-NA and p-NA substrates) activities. However, converging to what occurs in *C. pomonella* (Reyes et al, in press), these combined target site modification and detoxification mechanisms do not confer a high level of resistance to the analyzed pesticides. But in the case of these OFM populations, the activity ratios of the detoxifying enzymes were rather low, never exceeding two times more than the reference. The Brazilian populations also slightly differed from the reference strain for their specific AChE activity (0.4- to 0.7-fold) and for AChE's inhibition by carbaryl (1.7- to 2.5-fold in female moths). Comparatively, the 25-fold resistance to carbofuran of Canadian populations of *C. molesta* (Kanga et al. 1997) was associated to a close to 4-fold increase of a-NA and to a 1757- fold decrease of susceptibility of AChE to carbaryl, without any alteration of the MFO and GST activities. Further investigations

383 attributed this resistance to organophosphates and carbamates of North American populations of
384 *C. molesta* to a sex-linked alteration of AChE (Kanga et al. 1997, de Lame et al. 2001, Schearer
385 and Usmani 2001). The small differences of specific AChE activity and of inhibition by carbaryl
386 observed in our samples could also be the result of a mutation or of a modified expression of the
387 ace gene in some individuals. We therefore focused on qualitative or quantitative modifications
388 of the gene, more precisely on the section where a mutation MACE was described in *C.*
389 *pomonella* (Cassanelli et al. 2006). The sequencing did not reveal any modification in this part of
390 the gene, but a modification can be localised in another section of the gene. Moreover, only eight
391 insects could be sequenced at this step, and we thus have now to develop a routine test to screen
392 entire populations. Moreover, although the basic blueprint of life is encoded in DNA, the
393 execution of the genetic plan is carried out by the activities of proteins. The fabric of biological
394 diversity is therefore protein-based and natural selection acts at the protein and phenotypic level
395 (Karr 2008, Biron et al. 2010). Another hypothesis to test is that *C. molesta* resistance is caused
396 by post-transcriptional changes by using transcriptomics and proteomics tools (Biron et al. 2006,
397 Nedelkov et al. 2006, Karr 2008).

398
399 None of the observed mechanisms could explain the resistance to deltamethrin in Vacaria
400 population, which was significant when compared to the reference strain and with the two other
401 Brazilian populations as well. Compared to these two populations, Vacaria had the lowest GST
402 and MFO activities, and also expressed the lowest affinity for both EST substrates. Such specific
403 resistance to deltamethrin could thus be the result of a mutation in the target site of pyrethroids,
404 the voltage-dependant sodium channel, as previously demonstrated in numerous insect species
405 including *C. pomonella* (Brun Barale et al 2005, Reyes et al 2007) and also suspected in French

populations of *C. molesta* (Siegwart et al 2010a). The sequencing of this gene in *C. molesta* would thus be of particular interest, to go back to the conserved DNA samples of Brazilian and French populations.

The Lages population issuing from the orchard which was the most heavily treated during in the last five years had also the highest GST and EST activities, significantly higher than the Vacaria population, and had also an increased MFO activity when compared to the reference strain. Such activities were not related to an increased tolerance to any of the tested insecticides but conversely to a significantly increased susceptibility to tebufenozide. An increased susceptibility to thiacloprid was also recorded in Lages and Porto populations, without any relation with the observed mechanisms. Regarding the low resistance and activity ratios that were recorded in this study, it may be hypothesized that resistance mechanisms are at the beginning of the selection process, as attested by the large intra population variability and the high activities recorded in few individuals of the population issuing from the most heavily treated orchard in Lages.

Moreover, it cannot be excluded that a part of the resistance was lost during the few generations of rearing of the populations in the laboratory without selection pressure, due to the fitness cost usually associated to metabolic resistances (Roush and Plapp 1982, Mc Kenzie and Batterham 1991, Boivin et al. 2003) and to the AChE mutations as well (Bourguet et al 2004, Shi et al 2004). However this loss of resistance during the rearing process could only lead to an under estimation of the rate of resistant individuals in the analysed populations, without qualitative change in the observed mechanisms.

Establishing the baseline susceptibility to insecticides and the baseline activity of enzymatic metabolization systems is required for insecticide resistance monitoring and management (Roush and Tabashnik 1990). This was made possible with this study for insecticide groups which were

the most intensively used in apple orchards during previous decades, i.e. organophosphates, pyrethroids and carbamates. Due to their negative ecological impact, these compounds are now being replaced by more selective or new ones, including insect growth regulators and neonicotinoids. Despite the frequent observation of metabolic resistance to the moulting activator tebufenozide in various lepidopteran species (Sauphanor and Bouvier 1995, Smagghe et al. 1998, Waldstein and Reissig 2000, Cao & Han 2006), the efficacy of this insecticide was conserved against the tested populations. This is also the case for thiacloprid, which is not yet frequently applied in the studied area. The developed and tested method will be used to analyse new populations, trying now to get large collections allowing direct investigations on the F1 neonates of the sampled insects. This method will also be implemented for other insecticides, including the recent compounds that are expected to be registered in Brazil against *C. molesta*.

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Table 1: Susceptibility to five insecticides of neonate larvae of one reference strain (S_{it}) and three Brazilian populations (Vacaria, Porto, Lages) of *C. molesta*.

Insecticide	Population	N ²	LC50 ³ (95% CL)	LC50 susceptibility Ratio / S_{it} ⁴
Chlorpyrifos	S_{it} ¹	168	92.53 (40.70-177.44)	
	Vacaria	168	276.17 (171.28-568.68)	2.98 (2.23-3.99)
	Porto	165	272.44 (151.62-829.76)	2.94 (2.24-3.87)
	Lages	161	250.17 (134.35-814.38)	2.70 (2.06-3.54)
Deltamethrin	S_{it}	164	0.13 (0.10-0.18)	
	Vacaria	167	0.24 (0.17-1.72)	1.80 (1.16-2.79)
	Porto	166	0.12 (0.10-0.13)	0.88 (0.69-1.12)
	Lages	164	0.12 (0.11-0.14)	0.90 (0.75-1.09)
Thiacloprid	S_{it}	190	91.30 (72.60-108.46)	
	Vacaria	165	55.98 (17.57-78.65)	0.61 (0.42-0.90)
	Porto	168	39.39 (22.60-53.05)	0.43 (0.30-0.62)
	Lages	166	86.16 (66.92-110.40)	0.94 (0.67-1.34)
Tebufenozide	S_{it}	165	9.44 (7.18-10.94)	
	Vacaria	166	9.30 (5.44-11.62)	0.98 (0.71-1.36)
	Porto	188	8.15 (3.71-46.10)	0.86 (0.61-1.23)
	Lages	191	4.55 (2.99-7.49)	0.48 (0.36-0.64)
Carbaryl	S_{it}	191	165.3 (120.3-227.6)	
	Vacaria	189	237.3 (181.2-312.8)	1.44 (1.00-2.08)
	Porto	186	190.0 (120.7-229.9)	1.15 (0.77-1.71)
	Lages	188	144.4 (108.2-192.4)	0.87 (0.58-1.32)

¹ S_{it} – European reference strain mass-reared on artificial diet since over 10 years.

² Number of neonates tested.

³ LC₅₀ values expressed in mg L⁻¹.

⁴ Susceptibility Ratio/ S_{it} : Resistance ratio = LC50 of Brazilian strain divided by LC50 of the reference strain.

Table 2: Acetylcholinesterase inhibition by Carbaryl in adults of four populations of *C. molesta*.

Populations	Sex	n ^a	Slope ± SE	IC ₅₀ ^b (95% CI)	IR ^c (95% CI)	χ ²
S _{it}	Male	25	1.03 ± 0.07	0.012 (0.009 - 0.015)		3.8
	Female	25	0.84 ± 0.06	0.008 (0.006 - 0.011)		1.7
Vacaria	Male	25	1.15 ± 0.07	0.012 (0.010 - 0.015)	1.0 (0.9 - 1.2)	3.9
	Female	25	1.00 ± 0.07	0.011 (0.009 - 0.014)	1.7 (1.2 - 1.5)	4.3
Porto	Male	25	1.21 ± 0.09	0.017 (0.014 - 0.022)	1.5 (1.3 - 1.7)	1.8
	Female	25	1.07 ± 0.07	0.016 (0.012 - 0.020)	1.9 (1.7 - 2.1)	1.7
Lages	Male	25	1.15 ± 0.06	0.019 (0.015 - 0.024)	1.6 (1.5 - 1.8)	2.9
	Female	25	1.06 ± 0.07	0.021 (0.016 - 0.027)	2.5 (2.2 - 2.8)	4.6

^a Number of adult month tested.

^b Concentration are expressed in mmol/min/mg of protein for Carbaryl.

^c IC₅₀s estimated by probit analysis; inhibition ratio IR calculated by dividing the IC₅₀ for the Brazilian populations (Lages or Porto or Vacaria) by the IC₅₀ for our laboratory strain (S_{it}) for each gender.

Figure Legends

Figure 1: (a) Glutathion S Transferase activity, measured in fluorescence unit formed/min/ μ g of protein, in reference strains (S_{it}) and Brazilian strains (Vacaria, Porto and Lages) of the oriental fruit moth. (b) Carboxylesterase activity, measured in nmoles of product (α -Naphtol or p-Nitrophenol)/min/mg of protein. Letter (Capital one for esterase activity with p-NA and small letter for GST or esterase activity with α -NA) illustrate Tukey test ($p > 0.05$). (c) Acetylcholine esterase activity, measured in nmoles of acetylthiocholine/min/mg of protein, in reference strains (S_{it}) and brazillian strains (Vacaria, Porto and Lages) of the oriental fruit moth. Letter illustrate Tukey test ($p > 0.05$).

Figure 2: Distribution of MFO activities (pg of 7-HC/min/abdomen of insect) by populations and sex.

Figure 1

a)

b)

c)

Figure 2