Tools for Resistance Monitoring in Oriental Fruit Moth (Lepidoptera: Tortricidae) and First Assessment in Brazilian Populations

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ABSTRACT In southern Brazilian apple (*Malus* spp.) orchards, predominantly organophosphates are used to control the oriental fruit moth, *Cydia molesta* (Busck) (Lepidoptera: Tortricidae), 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 >10 yr. 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 with the reference strain. One of the field populations that 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 compared with the reference strain. All three populations had slight but significant increases of glutathione transferase and carboxylesterases activities and significant decrease of specific acetylcholinesterase activities compared with the reference. Only the most resistant population to chlorpyriphos exhibited a significantly higher mixed function oxidase activity than the reference. The acetylcholinesterase of females was significantly less inhibited by carbaryl in the Brazilian populations than in the reference strain (1.7–2.5-fold), and this difference was not expressed in the male moth. However, no mutation in the MACE locus was detected. These biological and molecular characterizations of adaptive response to insecticides in *C. molesta* provide tools for early detection of insecticide resistance in field populations of this pest.

RESUMO 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 seqüenciamento 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 freqüentemente 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 glutationa transferase e atividades carboxilesterases e diminuiram a atividade de acetilcolinesterase 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

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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.

KEY WORDS *Cydia molesta*, insecticide resistance, monitoring, mechanism, target mutation

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

C. molesta has been present in southern Brazil since the early 1980s (Lorenzato 1988), and its damage was first recorded in Vacaria (Rio Grande do Sul) and Fraiburgo (Santa Catarina) and then during the 1990s in São Joaquim (Santa Catarina) and Porto Amazonas (Paraná). The control of *C. molesta* in Brazil involved exclusively neurotoxic insecticides, including numerous organophosphates (OPs), until the end of 1990s. Tebufenozide and novaluron are the only insect growth regulators (IGRs) 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: Tephritidae) and *Bonagota cranaodes* (Meyrick) (Lepidoptera: Tortricidae) (Botton et al. 2000).

In Brazilian apple orchards and in southern European peach orchards as well, the protection against *C. molesta* requires up to 10 treatments per year (Monteiro et al. 2009, Siegwart et al. 2010b). Despite this high insecticide pressure, control failures were recorded in several locations of both production areas during the past 2 yr (Monteiro et al. 2009, Monteiro and Souza 2010, Siegwart et al. 2010b). This could be the result of the selection of insecticide resistance, as it has already occurred for OPs and carbamates in North American populations of *C. molesta* (Kanga et al. 1997, Pree et al. 1998).

Insecticide resistance management requires a discontinuous selection process that may be obtained from the use of nonchemical control methods (Roush and Tabashnik 1990, Monteiro et al. 2008) or by alternating insecticide compounds with different modes of action. The implementation of new compounds is thus of interest to counter the selection of resistance to neurotoxic compounds that may result from their intensive and often exclusive use in Europe (mainly pyrethroids) or in South America (mainly OPs). New neo-nicotinoid and insect growth regulator (IGR) families could be interesting tools, because several neurotoxic active ingredients are now withdrawn from European and South American registration.

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

In this study, the resistance status of Brazilian populations of *C. molesta* collected in more or less intensively sprayed orchards was investigated to five active ingredients from different insecticide families: 1) neonicotinoid, 2) pyrethroid, 3) molting activating compound (MAC), 4) carbamate, and 5) OP. Bioassays were paired with metabolic analysis and gene sequencing of insecticide molecular targets, to identify markers associated with the 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 transferases (GSTs), mixed function oxidases (MFOs), and carboxylesterases (ESTs) (Yasutomi 1983, Oppenoorph 1985). The total AChE activity, which may vary, resulting in resistance to OPs or carbamates (Zhu and Gao 1999) was estimated. Finally, genes coding for the two forms of AChE were partially sequenced. The whole methodology was designed to define molecular and multimetabolic diagnostic tools to investigate the combination of mechanisms involved in resistance phenomenon.

Materials 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 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 yr with two further introductions of new genetic material from the same orchard. This orchard had mainly been protected using pyrethroid and OP 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, whereas Lages received seven OPs and two MACs. 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 Institut National de la Recherche Agronomique (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 ± 1°C, 70% RH, and a photoperiod of 16:8 (L:D) h. Egg laying was obtained in plastic tubes (8 cm in diameter, 20 cm in length) closed on both sides with gaze. Eggs were placed on artificial diet (Guennelon et al. 1981) in a plastic container (30 by 14 by 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 yr in Crop Protection Service in Lyon, France, without any insecticide exposure.

Insecticides. Susceptibility of the populations to the OP chlorpyrifos-ethyl (Pyrinex ME, 250 g/liter, Makhteshim-Agan, Sevre, France), to the neonicotinoid thiacloprid (Calypso, 480 g/liter, Bayer CropScience, Lyon, France), to the pyrethroid deltametrin (Decis, 15 g/liter, Bayer CropScience), to the carbamate carbaryl (Sevin, 85% wettable powder, Certis, Guyancourt, France), and to the benzhydrazid tebufenozide (Confirm, 240 g/liter, Dow Agrosciences, Sophia Antipolis, France) were assessed. Fresh dilutions of formulated insecticides were prepared in distilled water for bioassays on neonates.

Bioassays. Microplate (96-wells, Sterilin, Dutscher SAS, Brumath, France) wells were filled with 150 μl of artificial diet (Stonefly Industries Ltd., Rochester, NY), and 6 μl of each insecticide solution was 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 d 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. GST, MFO, EST, and 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 analyzed for each enzymatic system. Fluorescence and absorbance were measured using a microplate reader (HTS 7000, PerkinElmer Life and Analytical Sciences, Boston, MA). 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 110 μl of 50 mM HEPES buffer, pH 7.0. For AChE activity, one head was homogenized on ice in 50 μl of 50 mM 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 0.05 M phosphate buffer, 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 according to Bradford (1976) by using bovine serum albumin to build the standard curve.

GSTs. GST activity was determined in black microplates (96-well, Corning Life Sciences, Lowell, MA) by using monochlorobimane (MCB) as substrate (Nauen and Stumpf 2002). The reaction mixture in one well consisted of 30 μl of enzymatic extract, 170 μl of a solution containing 6 μl of 100 mM glutathione, 162 μl of HEPES buffer (50 mM, pH 7.0), and 2 μl of 30 mM 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. Because the bimane-glutathione adduct was not commercially available, the activity was expressed as fluorescence units per minute per microgram of total protein extracted.

ESTs. Total nonspecific EST activity was measured with α-naphthyl acetate (α-NA) and para nitrophenyl acetate (p-NA) as substrates (Reyes et al. 2011). The reaction mixture for the first substrate 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. The reaction was stopped and colored after 20-min incubation at 22°C in darkness, by adding 55 μl of 0.2% Fast garnet GBC in 2.5% sodium dodecyl sulfate solution. Absorbance was recorded at 590 nm, after incubation during 20 min in darkness at room temperature.

The reaction mixture for the second substrate was 2.5 μl of protein extract, 2.5 μl of p-NA (50 mM in dimethyl sulfoxide) in 245 μl of HEPES buffer (50 mM, pH 7.0) with EDTA (1 mM) in each well. Absorbance was recorded at 405 nm each minute (kinetic mode) during 10 min. If the stabilization of speed reaction occurred, the difference of optical density per minute was calculated; otherwise, the measurement was repeated. Two standard curves with α-napthol (0–18 nmol per well) or p-nitrophenol (0–37.5 nmol per well) were elaborated to express activity in nanomoles of product per minute per milligram of total proteins.

MFOs. The MFO activity was determined using 7-ethoxycoumarin *O*-deethylation (ECOD) (Ulrich and Weber 1972) adapted for in vivo analysis in microplate. Twenty-four fresh insects of each strain were analyzed. Adult abdomens were dissected and homogenized in incubation solution (100 μl of HEPES buffer

[50 mM, pH 7.0] with 7-ethoxycoumarin [0.4 mM]) on ice and centrifuged at 15 000 × *g* for 2 min at 2°C. Supernatants were individually placed in wells of black microplates (96-well, Corning Life Sciences). After 4-h incubation at 30°C, the reaction was stopped by adding 100 μl of 1.5 M 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 picograms of 7-HC per insect per min via to a standard curve of 7-HC (0.5–4.5 nmol per well).

AChE. The AChE activity was determined using acetylthiocholine (ASCh) as substrate (Ellman et al. 1961). To analyze 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:

$$\text{ASCh} \xrightarrow{\text{(enzyme)}} \text{thiocholine} + \text{acetate}$$

$$\text{thiocholine} + \text{dithiobisnitrobenzoate (DTNB)} \longrightarrow \text{thionitrobenzoate (yellow color)}$$

Nineteen to 40 insect extracts were analyzed per sex and population. The reaction mixture in one well was composed of 138.5 μl of phosphate buffer (0.1 M, pH 8.0), 5 μl of DTNB (0.01 M, 0.3 mM final concentration), 1.5 μl of ASCh (0.1 M, 1 M final concentration), and 5 μl of protein extract. Four wells receiving phosphate buffer (0.1 M, pH 8.0) 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 nanomoles of thiocholine per minute per milligram of total protein via standard curves with DTT (0–4.5 nmol per well) instead of thiocholine (Ellman et al. 1961). Because 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. We dissolved 20 μl of carbaryl in ethanol 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, 10 μl of extracts was incubated at 4°C during 20 min. In the control wells the insecticide was replaced by the solvent alone and in the blank by 0.01 M eserine. The reaction was initiated by addition of a solution containing 133.5 μl of phosphate buffer (0.1 M, pH 8.0), 5 μl of DTNB (0.01 M, 0.3 mM final concentration), and 1.5 μl of ASCh (0.1 M, 1 mM 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 (IC_{50}) of AChE activity were estimated though probit analysis (Russell et al. 1977). Differences in inhibition among genotypes were considered not significant if the 95% confidence limits (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 1,200 ppm of chlorpirifos. Theses last two individual are used to sequence ace2 gene.

We performed the total DNA extraction of *C. molesta* by using a hexadecyl-trimethyl-ammonium bromide (CTAB) protocol (Murray and Thompson 1980). Adults were individually ground in 200 μl of proteinase K (0.3 mg/ml) and incubated overnight at 56°C. Lysis occurred at 65°C during 1 h by adding 300 μl of lysis buffer (200 mM Tris-HCl, 50 mM EDTA, 2 M NaCl, and 2% CTAB) and 100 μl of Sarcosyl 5%. Proteins were precipitated and separated by chlorophorm:isoamylalcohol (24:1) treatment. Nucleic acids were precipitated at −20°C after adding 1 volume of isopropanol. The DNA pellet was washed in ethanol and resuspended in 60 μl of water. Polymerase chain reaction (PCR) amplifications were carried out in a 25-μl reaction volume containing 1× GoTaq buffer (Promega, Madison, WE), 200 μM each dNTPs, 0.4 μM each primer, 1 U of GoTaq, and 2 μl of DNA template.

For amplification in the first gene of AChE (ace1), we used the 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′-tgccttccttcatcattgtg-3′).

Thermal conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 60 s, and 74°C for 2 min. 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 accession DQ267977 (Cassanelli et al. 2006). For ace2, the cDNA sequence of the gene (accession HM775184) was provided by S. Cassanelli (University of Modena and Reggio Emilia, Italy).

The PCR fragments obtained were purified after visualization using 1% agarose gel with the QIAquick gel extraction kit (Genome Express, Meylan, France) and directly sequenced. Data analysis was performed with the BioEdit software (Tom Hall, Carlsbad, CA).

Statistical Analyses. A probit analysis on corrected mortalities (Abbott 1925) was done to determine the LC_{50} values (Raymond 1985). Resistance ratios at the LC_{50} (RR_{50}, the ratio between the LC_{50} of the resistant strain and the LC_{50} of the susceptible strain) and their 95% CL were calculated. The LC_{50} values were considered different when the 95% CL of their RR_{50} did not include 1. Biochemical data were subjected to analysis of variance. Means were compared by the protected least significant difference Tukey test ($P < 0.05$) by using the software R (Bell Laboratories, Murray Hill, NJ).

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	Pop	n^a	$LC_{50}{}^b$ (95% CL)	LC_{50} susceptibility ratio/$S_{it}{}^c$
Chlorpyriphos	$S_{it}{}^d$	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)

[a] Number of neonates tested.
[b] LC_{50} values expressed in milligrams per liter^{-1}.
[c] Susceptibility ratio/S_{it}: resistance ratio = LC_{50} of Brazilian strain divided by LC_{50} of the reference strain.
[d] S_{it}, European reference strain mass reared on artificial diet since >10 yr.

Results

Laboratory Bioassays. The three Brazilian populations tested were significantly less susceptible to chlorpyriphos than the European reference strain S_{it}, with RR_{50} values ranging from 2.70 to 2.98 (Table 1). The Vacaria population was also significantly resistant to deltamethrin (RR_{50} = 1.80), whereas the two other populations were not. Vacaria population also was the least 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 2 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 chlorpyriphos ethyl. However, the Vacaria population was two-fold less susceptible to deltamethrin than Porto and Lages (Vacaria versus Porto RR_{50} = 2.04 [1.32–3.16]; Vacaria versus 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 Sit strain (F = 34.11, df = 39, $P < 0.001$; F = 25.34, df = 43, $P < 0.001$; and F = 15.95, df = 45, $P < 0.001$ for Lages, Porto, and Vacaria, respectively) (Fig. 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 (Fig. 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, $P < 0.001$; and 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, $P < 0.001$; and 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, whereas 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 AChE 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) (Fig. 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, $P < 0.002$) (Fig. 2), due to their significant bigger size (data not shown). This difference was verified regardless of 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, $P < 0.005$; and Lages: T = 8.2, df = 22, $P < 0.001$). The inhibition of the AChE activity by carbaryl was significantly lower in the Brazilan 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 AChE. Parts of the two AChE genes (ace1 and ace2) were sequenced for the first time in *C. molesta* to search for a mutation potentially involved in OP 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). Seventy-four point 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

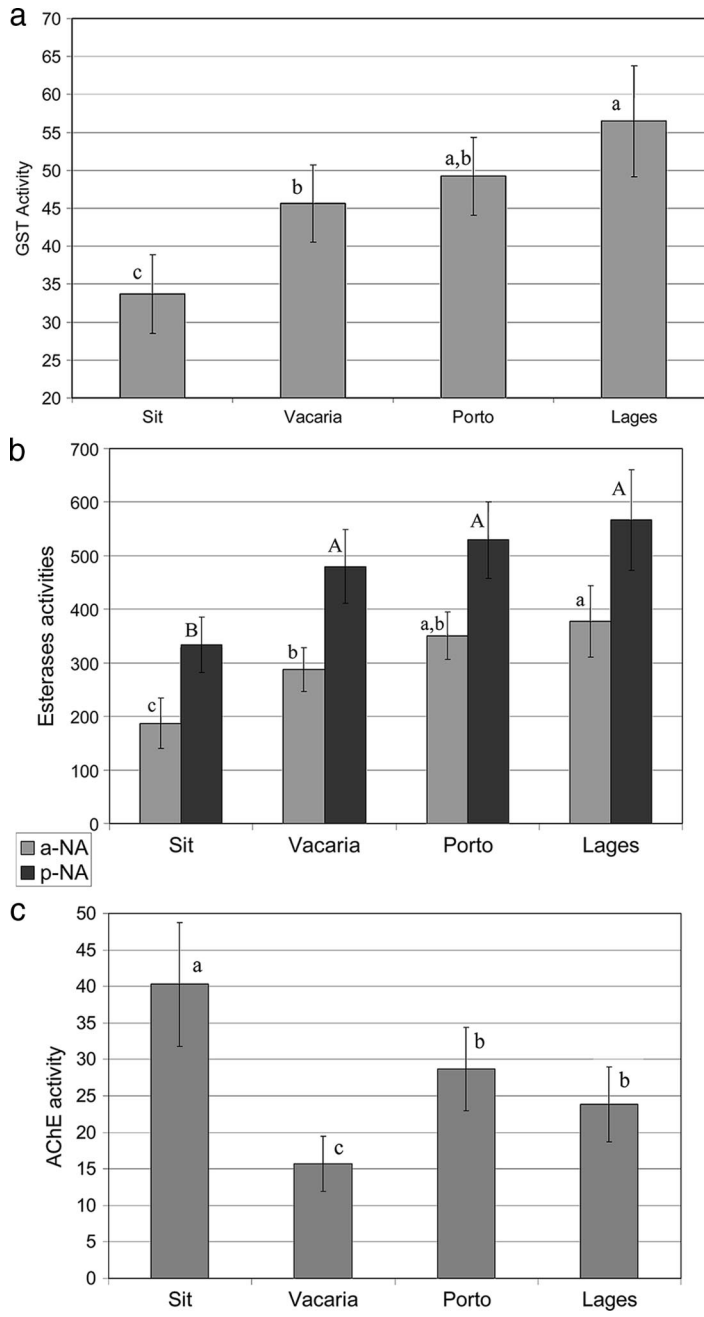

Fig. 1. (a) GST activity, measured in fluorescence unit formed per minute per microgram of protein, in reference strains (S_{it}) and Brazilian strains (Vacaria, Porto, and Lages) of the oriental fruit moth. (b) Carboxylesterase activity, measured in nanomoles of product (α-naphtol or p-nitrophenol) per minute per milligram of protein. Letters (uppercase for esterase activity with p-NA and loswercase for esterase activity with α-NA) illustrate Tukey's test results ($P > 0.05$). (c) AChE activity, measured in nanomoles of ASCh per minute per milligram of protein, in reference strains (S_{it}) and Brazilian strains (Vacaria, Porto, and Lages) of the oriental fruit moth. Letters illustrate Tukey's test results ($P > 0.05$).

variant amino acids exist in other species. They are presumably not involved in the enzyme conformation and activity. The second gene knows to code AChE in *Cydia* genus, ace2 was partially sequenced in individuals showing high tolerance to chlopyrifos in our bioassays (AN in GenBank HM775184). But none sequences differences was found between our S_{it} strain and these individuals.

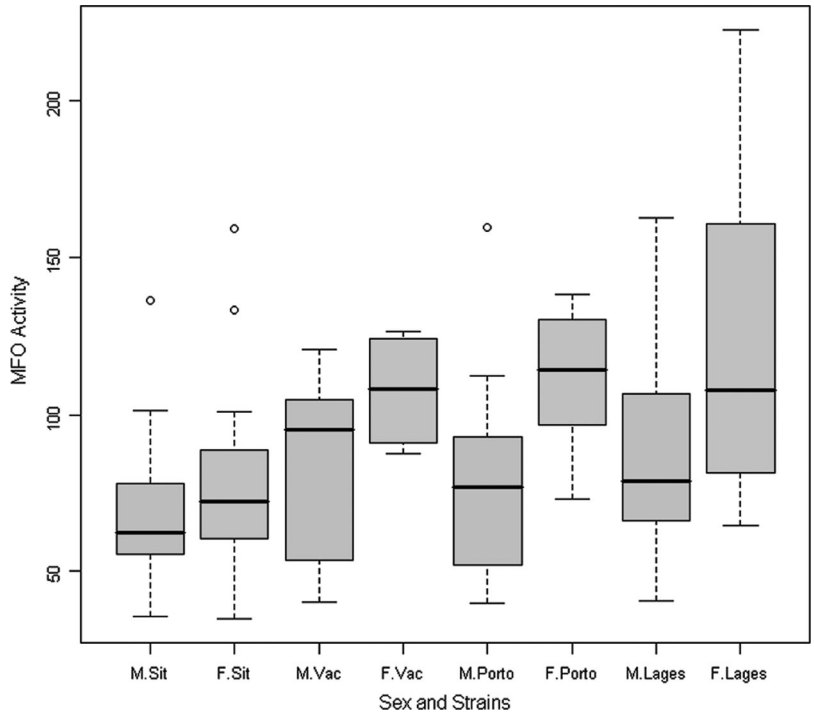

Fig. 2. Distribution of MFO activities (picograms of 7-HC per minute per abdomen of insect) by populations and sex.

Discussion

The first aim of this study was to set up in *C. molesta* a method developed previously 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 metabolization, and sequencing of the molecular target of OPs.

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 chlopryriphos than the reference strain S_{it}, in coherence with the frequent applications of OP insecticides in Brazilian orchards to prevent *C. molesta* injury. Such reduced susceptibility to OPs may partially explain the heavy damages on fruit caused in these orchards by C. *molesta* during the past 5 yr. However, Vacaria population was significantly more tolerant to deltamethrin than Porto and Lages, probably because pyrethroïds were more frequently used before 2000 than now. 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.

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

Pop	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 moths tested.
[b] Concentration are expressed in millimoles per minute per milligram of protein for carbaryl.
[c] IC_{50} values estimated by probit analysis; inhibition ratio IR was calculated by dividing the IC_{50} for the Brazilian populations (Lages, Porto, or Vacaria) by the IC_{50} for our laboratory strain (S_{it}) for each gender.

In our study, the reliability of the reference strain has to be considered carefully. It was collected in Italy >15 yr 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 responses to insecticide and the enzyme activities were often observed to be dependent on the host plant, as demonstrated for the susceptibility of *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae) to OPs (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 chlorpyriphos compared with 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 also was the least susceptible to carbaryl. Similar OP resistance associated to reduced AchE's affinity for ASCh substrate was described previously in *C. pomonella* (Reuveny and Cohen 2004). This resistance to chlorpyriphos in oriental fruit moth populations also was associated with an increase of GST and EST (for both α-NA and p-NA substrates) activities. However, converging to what occurs in *C. pomonella* (Reyes et al. 2011), these combined target site modification and detoxification mechanisms do not confer a high level of resistance to the analyzed pesticides. But in these oriental fruit moth populations, the activity ratios of the detoxifying enzymes were rather low, never exceeding 2 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 fourfold increase of a-NA esterase activity and to a 1,757-fold decrease of susceptibility of AChE to carbaryl, without any alteration of the MFO and GST activities. Further investigations attributed this resistance to OPs and carbamates of North American populations of *C. molesta* to a sex-linked alteration of AChE (Kanga et al. 1997, de Lame et al. 2001, Shearer and Usmani 2001). The small differences of specific AChE activity and of inhibition by carbaryl observed in our samples also could be the result of a mutation or of a modified expression of the ace gene in some individuals. We therefore focused on qualitative or quantitative modifications of the gene, more precisely on the section where a mutation MACE was described in *C. pomonella* (Cassanelli et al. 2006). The sequencing did not reveal any modification in this part of the gene, but a modification can be localized in another section of the gene. Moreover, only eight insects could be sequenced at this step, and we thus have now to develop a routine test to screen entire populations. Moreover, although the basic blueprint of life is encoded in DNA, the execution of the genetic plan is carried out by the activities of proteins. The fabric of biological diversity is therefore protein-based and natural selection acts at the protein and phenotypic level (Karr 2008, Biron et al. 2010). Another hypothesis to test is that *C. molesta* resistance is caused by posttranscriptional changes by using transcriptomics and proteomics tools (Biron et al. 2006, Nedelkov et al. 2006, Karr 2008).

None of the observed mechanisms could explain the resistance to deltamethrin in Vacaria population, which was significant compared with the reference strain and with the two other Brazilian populations as well. Compared with these two populations, Vacaria had the lowest GST and MFO activities and also expressed the lowest affinity for both EST substrates. Such specific resistance to deltamethrin could thus be the result of a mutation in the target site of pyrethroids, the voltage-dependant sodium channel, as demonstrated previously in numerous insect species, including *C. pomonella* (Brun-Barale et al. 2005, Reyes et al. 2007), and also is suspected in French populations of *C. molesta* (Siegwart et al. 2010a). The sequencing of this gene in *C. moletsa* 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 that was the most heavily treated during in the past 5 yr also had the highest GST and EST activities, significantly higher than the Vacaria population, and it also had an increased MFO activity compared with 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 also was 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, McKenzie 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 analyzed 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 that were the most intensively used in apple orchards during previous decades, i.e., OPs, pyrethroids, and carbamates. Due to their negative ecological impact, these compounds are now being replaced by more selective or new compounds, including IGRs and neo-nicotinoids. Despite the frequent observation of metabolic resistance to the molting activator tebufenozide in various lepidopteran species (Sauphanor and Bouvier 1995, Smagghe et al. 1998, Waldstein and Reissig 2000, Cao and 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 analyze new populations, trying now to get large collections allowing direct investigations on the F1 neonates of the sampled insects. This method also will be implemented for other insecticides, including the recent compounds that are expected to be registered in Brazil against *C. molesta.*

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