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

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3 **Tools for resistance monitoring in the oriental fruit moth (Lepidoptera: Tortricidae) and**
4 **first assessment in Brazilian populations**
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15 # Abstract

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

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

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38 # Abstract in Portuguese

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

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

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61 The oriental fruit moth, *Cydia molesta* (Busk), is a damaging lepidopteran pest of peach tree
62 (*Prunus persicae* (Linné)) orchards. *C. molesta* larvae attack both shoots and fruits of its native
63 host plant, but this moth is also known to feed on quince (*Cydonia oblonga* (Mill.)), apricot
64 (*Prunus armeniaca* (Linné)), almond (*Prunus dulcis* (Mill.)), medlar (*Mespilus germanica*
65 (Bosc.)), and to a minor degree on cherry (*Prunus cerasus* (Linné)) and plum (*Prunus domestica*
66 (Linné)) (Balachowsky 1966). Moreover, while this species was previously expected to migrate
67 towards pome fruits in the late generations after the harvest of stone fruits, *C. molesta* has
68 acquired the ability to complete its life cycle on American and European pome fruit orchards and
69 is causing heavy damages on apple and pears (Reis et al. 1988, Usmani and Shearer 2001, Natale
70 et al. 2003).
71 In South Brazil *C. molesta* is present since the early 1980s (Lorenzato 1988), and its damages
72 were first recorded in Vacaria (Rio Grande do Sul) and Fraiburgo (Santa Catarina) then during
73 the 90's in São Joaquim (Santa Catarina) and Porto Amazonas (Paraná). The control of *C.*
74 *molesta* in Brazil involved exclusively neurotoxic insecticides including numerous
75 organophosphates until the end of 1990s. Tebufenozide and novaluron are the only insect growth
76 regulators registered against *C. molesta* in Brazil (Andrei 2009). These insecticides are aimed to
77 control simultaneously *C. molesta* and secondary pests, including *Anastrepha fraterculus*
78 (Wiedemann) (Diptera: Tethritidae) and *Bonagota cranaodes* (Meyrick) (Lepidoptera:
79 Tortricidae) (Botton et al. 2000).
80 In Brazilian apple orchards and in South-European peach tree orchards as well, the protection
81 against *C. molesta* requires up to ten treatments per year (Monteiro et al. 2009, Siegwart 2010).
82 Despite this high insecticide pressure, control failures were recorded in several locations of both
83 production areas during the last two years (Monteiro and Souza 2010, Monteiro et al. 2009,

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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 necotinoid 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

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

115 **Material and methods**

116 **Insects**

117 Three Brazilian populations of *C. molesta* were sampled in apple orchards in Vacaria (Rio
118 Grande do Sul state), Lages (Santa Catarina state), and Porto Amazonas (Paraná state). A
119 susceptible laboratory strain (S_{it}) originating from Italian peach tree orchards (Provided by Fabio
120 Molinari, University of Piacenza, Italy) was used as reference. The Vacaria population was
121 collected in 2005 and reared in the laboratory on artificial diet (Guennelon et al. 1981) during 3
122 years with two further introductions of new genetic material from the same orchard. This orchard
123 had mainly been protected using pyrethroid and organophosphate applications previous to
124 collecting the population. The other two Brazilian populations were field collected in early 2008
125 in orchards where failure of chemical protection occurred. In 2006/2007 season five OPs were
126 applied in Porto orchard while Lages received seven organophophates and two MAC. Close to
127 10% damages were recorded in 2007 in Lages orchard despite this protection program
128 (unpublished data). Porto and Lages populations were reared in Parana Federal University,
129 Curitiba (Brazil) during two generations. The three Brazilian populations were then transferred at

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130 INRA Avignon, where analyses were performed. These analyses were completed during three
131 successive generations, required to multiply the insects and for preliminary tests to set the
132 adequate concentrations of the different insecticide and the methodology of enzyme analysis.
133 The rearing of the field populations and of the reference strain was conducted at 25±1°C, 70%
134 RH under 16 L: 8 D photoperiod. Egg laying was obtained in plastic tubes (8cm in diameter, 20
135 cm long) closed on both sides with gaze. Eggs were placed on artificial diet (Guennelon et al
136 1981) in a plastic container (30 x 14 x 10 cm) for larval development. Larvae pupated in gaze
137 strips, in plastic containers. The reference strain S_{it} had been mass reared on artificial diet for 10
138 years in Crop Protection Service of Lyon (France), without any insecticide exposure.

139 **Insecticides**

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

146 **Bioassays**

147 Microplate (96-wells, Sterilin®) wells were filled with 150 µL of artificial diet (Stonefly
148 Industries Ltd, Rochester, NY), and 6 µL of each insecticide solution were applied to the diet's
149 surface according to Reyes and Sauphanor (2008). For each population, concentration-response
150 relationships were established per insecticide. Six concentrations giving between 0 and 100%
151 mortality were used. Distilled water replaced insecticide in the control (Fuentes-Contreras et al.
152 2007). Newly hatched larvae (0–4 h old) were individually placed in the wells. Mortality was

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

156 **Enzymatic activities**

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

164 **Enzyme extracts.** EST and GST activities were evaluated using single thorax homogenized in
165 110µl of 50mM Hepes buffer (pH 7). For AChE activity, one head was homogenized on ice in
166 50µl of 50mM phosphate buffer with 0.5% Triton (pH 7.2). For AChE inhibition five heads of
167 adults were pooled and homogenized in 250 µl of ice-cold phosphate buffer (0.05M; pH 7.2)
168 containing 0.5% Triton. The homogenates obtained from each insect segment were centrifuged at
169 15 000 × g for 15 min at 4°C, and the supernatants were used as enzyme sources (Bouvier et al.
170 2002). The protein content of each sample was measured (Bradford 1976) using bovine serum
171 albumin to build the standard curve.

172 **Glutathione S-transferases.** GST activity was determined in black microplates (96-wells,
173 Costar®) using monochlorobimane (MCB) as substrate (Nauen and Stumpf 2002). The reaction
174 mixture in one well consisted of 30µl of enzymatic extract, 170µl of a solution containing: 6µl of
175 100mM glutatione (GSH), 162µl of Hepes buffer (50mM, pH7.0) and 2µL of 30mM MCB.

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176 Wells with Hepes buffer instead of enzyme extract were used as controls. Fluorescence was
177 measured after 20 min incubation at 22°C, with 380 nm excitation and 450 nm emission filters.
178 Since the bimane-glutathione adduct was not commercially available, the activity was expressed
179 as fluorescence units per min per µg of total protein extracted.
180 **Esterases.** Total non-specific EST activity was measured with α-naphthyl acetate (α-NA) and
181 para nitrophenyl acetate (p-NA) as substrates (Reyes et al, in press). The reaction mixture for the
182 first substrate was 1µl of protein extract and 194µl of 30µM α-NA in Hepes buffer (50mM, pH
183 7.0) in each microplate well. The reaction was stopped and coloured after 20 min incubation at
184 22°C in darkness, by adding 55µl of 0.2% Fast garnet GBC in 2.5% sodium dodecyl sulphate
185 solution. Absorbance was recorded at 590nm, after incubation during 20 min in darkness at room
186 temperature.
187 The reaction mixture for the second substrate was 2.5µl of protein extract, 2.5µl of p-NA (50
188 mM in DMSO) in 245µl of Hepes buffer (50 mM, pH 7) with EDTA (1 mM) in each well.
189 Absorbance was recorded at 405nm each minute (kinetic mode) during 10min. If the stabilisation
190 of speed reaction occurred, the difference of OD per min was calculated, otherwise the
191 measurement was repeated. Two standard curves with α-Naphtol (0-18nmoles/well) or p-
192 nitrophenol (0-37.5nmoles/well) were elaborated to express activity in nmoles of
193 product/min/mg of total proteins.
194 **Mixed-function oxidases.** The MFO activity was determined using 7-ethoxycoumarin O-
195 deethylation (ECOD) (Ulrich 1972) adapted for in vivo analysis in microplate. Twenty four fresh
196 insects of each strain were analysed. Adult abdomens were dissected and homogenised in
197 incubation solution: 100µl of Hepes buffer (50mM pH 7) with 7-ethoxycoumarin (0.4mM); on
198 ice and centrifuged at 15 000 × g for 2 min at 2°C. Supernatants were individually placed in

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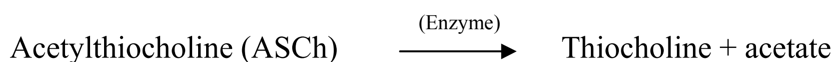
199 wells of black microplates (96-wells, Costar®). After 4h incubation at 30°C, the reaction was
200 stopped by adding 100µL of 1.5M glycine buffer (pH 10.3). The 7-hydroxycoumarin (HC)
201 fluorescence was quantified with 380 nm excitation and 465 nm emission filters. Four wells
202 receiving glycine buffer previous to incubation were used as control. The activity was expressed
203 as pg of 7-HC/insect/min thanks to a standard curve of 7-Hydroxycoumarine (0.5-4.5
204 nmoles/well).
205 **Acetylcholine esterase.** The AChE activity was determined using acetylthiocholine (ASCh) as
206 substrate (Ellman et al. 1961). To analyse numerous samples, Ellman's protocol was adapted for
207 microplate analysis. The enzyme activity was measured by increase of yellow coloration due to
208 the formation of thionitrobenzoate, based on the following reactions:
209 
Acetylthiocholine (ASCh) —(Enzyme)→ Thiocholine + acetate
210
Thiocholine + Dithiobisnitrobenzoate (DTNB) → Thionitrobenzoate (yellow color)
211 Nineteen to 40 insect extracts were analysed per sex and population. The reaction mixture in one
212 well was composed of 138.5µl of phosphate buffer (0.1M, pH 8.0), 5µl of DTNB 0.01M (0.3
213 mM final concentration), 1.5µl ASCh 0.1M (1M final concentration) and 5µl of protein extract.
214 Four wells receiving phosphate buffer (0.1M, pH 8) instead of protein extract were used as
215 control. Absorbance was recorded at 405 nm every minute (kinetic mode) during 10 min. When
216 the reaction speed was stabilized, the activity was calculated and expressed in nmoles of
217 Thiocholine/min/mg of total protein thanks to a standard curves with DTT (0-4.5 nmoles/well)
218 instead of thiocholine (Ellman et al. 1961). As DTT has two functional groups -SH this standard
219 curve has to be divided by two before using.
220 The inhibition of AChE activity by carbaryl was assessed using 25 insects per sex and
221 population. 20µl of carbaryl dissolved in ethanol was placed in each well of a microplate, and the

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

233 **Sequencing the molecular targets of insecticides**

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

237 We performed the total DNA extraction of *C molesta* using a hexadecyl-trimethyl-ammonium
238 bromide (CTAB) protocol (Murray and Thompson 1980). Adults were individually ground in
239 200µl of proteinase K (0.3 mg/ml) and incubated over night at 56°C. Lysis occured at 65°C
240 during 1h by adding 300µl of lysis buffer (Tris-HCl 200mM, EDTA 50mM, NaCl 2M, CTAB
241 2%) and 100µl of sarcosyl 5%. Proteins were precipitated and separated by chlorophorm-
242 isoamylalcohol (24:1) treatment. Nucleic acids were precipitated at -20°C after adding one
243 volume of isopropanol. DNA pellet was washed in ethanol and resuspended in 60µl of water.
244 PCR amplifications were carried out in a 25µL reaction volume containing, 1X GoTaq buffer

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245 (Promega), 200µM of each dNTPs, 0.4µM of each primer, one Unit of GoTaq and 2µl of DNA
246 template.
247 For amplification in the first gene of AChE (ace1), we used primers: ACE 1S (5'-
248 cccagacctgttgaaagctg-3') and ACE 1R (5'-tgctctctggtaatgcctacg-3') and for amplification in the
249 second gene (ace2) we used primers: ACE 2S (5'-gtgccgcagcatttaagagt-3') and ACE 2R (5'-
250 tgccttccttcatcattgtg-3')
251 Thermal conditions were : 94°C for 3min, followed by 35 cycles of 94°C for 30s, 55°C for 60s
252 and 74°C for 2min. For ace1, primers were designed using a sequence of the related tortricid
253 moth Cydia Pomonella L (Lepidoptera: Tortricidae). This sequence name is cydpom-ace1, noted
254 in the GenBank database under the accession number DQ267977 (Cassanelli et al. 2006). For
255 ace2 the cDNA sequence of the gene (accession number: HM775184) was provided by S.
256 Cassanelli (University of Modena and Reggio Emilia, Italie).
257 The PCR fragments obtained were purified after visualisation using 1% agarose gel with the
258 QIAquick® Gel Extraction Kit and directly sequenced (Genome express, Meylan, France). Data
259 analysis was performed with the BioEdit software (Tom Hall, Carlsbad, US).
260 **Statistical analyses**
261 A probit analysis on corrected mortalities (Abbott 1925) was done to determine the LC50 values
262 (Raymond 1985). Resistance ratios at the LC50 (RR_{50}, which is the ratio between the LC50 of
263 the resistant strain and the LC50 of the susceptible strain) and their 95% CL were calculated. The
264 LC50s were considered different when the 95% CL of their RR_{50} did not include 1. Biochemical
265 data were subjected to analysis of variance (ANOVA). Means were compared by the protected
266 least significant difference Tukey test ($P < 0.05$) using the software R (Bell Laboratories, Murray
267 Hill, US).

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268 **Results**

269 **Laboratory bioassays**

270 The three Brazilian populations tested were significantly less susceptible to chlorpyriphos than
271 the European reference strain S_{it}, with RR_{50} ranging from 2.70 to 2.98 (Table 1). The Vacaria
272 population was also significantly resistant to deltamethrin (RR_{50}=1.80) while the two other
273 populations were not. Vacaria population was also the less susceptible to carbaryl, with 1.00 –
274 2.08 values of the 95% CL of its RR_{50} (Table 1). Surprisingly, two populations were significantly
275 less susceptible to thiacloprid than the reference strain (RR_{50}=0.61 and 0.43 for Vacaria and
276 Porto, respectively), and the Lages population was two times less susceptible to tebufenozide
277 than S_{it} (RR_{50}=0.48).
278 Despite differences in their selection pressure, the three Brazilian codling moth populations did
279 not exhibit strong differences in their insecticide susceptibility. All of them responded similarly
280 to chlorpyriphos ethyl. However the Vacaria population was 2-fold less susceptible to
281 deltamethrin than Porto and Lages (Vacaria vs Porto RR_{50}= 2.04 (1.32 -3.16); Vacaria vs Lages
282 RR_{50}=1.96 (1.27 – 3.05)). In the same way, Porto population was 2.19 (1.60 – 2.99) times more
283 susceptible to thiacloprid than Lages, and Lages was 2.04 (1.53 – 2.72) times more susceptible to
284 tebufenozide than Vacaria .
285

286 **Enzymatic activities**

287 The three Brazilian populations exhibited significantly higher GST activities than the Sit strain
288 (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
289 Lages, Porto and Vacaria, respectively) (Figure 1a). The highest enzymatic ratio was obtained
290 between Lages and S_{it} strain (1.7 fold). No sex-linked variability was observed for this enzyme

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291 system ($F = 2.309$, df = 1, $P = 0.133$) (Data not shown). EST activities measured using both α-
292 NA and p-NA substrates followed similar patterns as GST activity (Figure 1b), with significantly
293 higher values in the three Brazilian populations than in the S_{it} strain ($F = 29.060$, df = 40, $P <$
294 0.001; $F = 35.112$, df = 44, $P < 0.001$; $F = 14.914$, df = 46, $P < 0.001$ with α-NA for Lages,
295 Porto and Vacaria, respectively; $F = 31.400$, df = 39, $P < 0.001$; $F = 29.863$, df = 43, $P < 0.001$;
296 $F = 17.512$, df = 43, $P = 0.001$ with p-NA for Lages, Porto and Vacaria, respectively). The
297 highest enzymatic ratio was between Lages and S_{it} strain: 2.0 fold for α-NA and 1.7 fold for p-
298 NA. The Lages populations, collected in orchards where failure of chemical protection occurred,
299 had higher GST and a-NA activities than the Vacaria population while the Porto population
300 exhibited intermediate activities for both enzyme systems. These three populations did not differ
301 significantly from each other for p-NA EST activities.
302 A different pattern was observed for specific acetylcholine esterase activity, with a significantly
303 higher activity in the S_{it} strain than in Brazilians populations ($F = 87.4077$, df = 211, $P < 0.001$).
304 But as for GST and EST enzyme systems, the Vacaria population exhibited a lower specific
305 AChE activity than Porto and Lages ($F = 50.9626$, df = 105, $P < 0.0001$ and $F = 27.4031$, df =
306 126, P<0.0001, respectively) (Figure 1c). No sex-linked variability was observed for AChE
307 activities ($F = 0.375$, df =211, $P = 0.5410$,).
308 Only the Lages population had significantly higher MFO activity than the S_{it} strain ($F = 7.7$, df =
309 46, $P = 0.008$,). Moreover, activity ratio is low (1.4-fold), and the three Brazilian populations did
310 not differ significantly from each other for this character. All tested females exhibited a higher
311 MFO activity than males ($F = 11.3$, df = 84, $P < 0.002$) (Figure 2), due to their significant bigger
312 size (data not shown). This difference was verified whatever the population (S_{it}: T = 7.9, df =22,
313 $P < 0.001$; Porto: T = 18.2, df = 22, P 0.001; Vacaria: T = 10.9, df = 15, $P < 0.005$; Lages: T =

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314 8.2, df = 22, $P < 0.001$). The inhibition of the AchE activity by carbaryl was significantly lower
315 in the Brazilan population than in the S_{it} strain, excepted for the males of the Vacaria population
316 (Table 2). The AChE of Lages population was also less susceptible to the inhibition by carbaryl
317 than that of Vacaria moths.

318 **Gene sequencing of acetylcholinesterase**

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

330

331 **Discussion**

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

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337 The microplate bioassay on neonates already described for *C. pomonella* (Reyes and Sauphanor
338 2008) proved appropriate for *C. molesta* and provided reliable results for the five tested
339 insecticides, highlighting small but significant differences between the tested populations and the
340 reference susceptible strain. The three Brazilian populations exhibited a higher tolerance to
341 chlopryriphos than the reference strain S_{it}, in coherence with the frequent applications of
342 organophosphorous insecticides in Brazilian orchards to prevent *C. molesta* injury. Such reduced
343 susceptibility to organophosphates may partially explain the heavy damages on fruits caused in
344 these orchards by C. *molesta* during the last five years. On the other hand, Vacaria population
345 was significantly more tolerant to deltamethrin than Porto and Lages, probably due to the fact
346 that pyrethroïds were more frequently used before the year 2000 than nowadays. Indeed Vacaria
347 is one of the oldest and the largest growing area for apple orchards in Brazil, planted in the
348 beginning of 1980. Lages and Porto orchards were planted more recently, in 1990 and 1998,
349 respectively, and therefore received only few pyrethroid treatments during their production
350 period.
351
352 In our study the reliability of the reference strain has to be considered carefully. It was collected
353 in Italy over 15 years ago in an untreated peach tree orchard, and was then maintained in
354 continuous mass rearing in the laboratory without any selection pressure. It is thus expected to be
355 susceptible to insecticides, but also to have a genetic background strongly different from the
356 populations more recently sampled, in apple orchards of a distant continent. All our populations
357 had been reared on artificial diet for a few generations in the same conditions previous to the
358 study, avoiding the differential induction of digestive enzymes by the host plants. However we
359 cannot exclude that the differences of enzyme activities recorded between the field populations

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360 and the reference result from these distant genetic backgrounds rather than from an adaptive
361 response to the insecticide pressure. The response to insecticide and the enzyme activities were
362 often observed to be under dependence on the host plant, as demonstrated for the susceptibility
363 of *Epiphyas postvittana* (Lepidoptera: Tortricidae) to organophosphates (Robertson, et al. 1990).
364 The comparison of the responses to insecticides and of biochemical characteristics between the
365 three Brazilian populations is thus meaningful.
366
367 The three Brazilian populations exhibited a reduced susceptibility to chlorpyriphos compared to
368 the reference strain, together with a decreased specific activity of the AChE target of Ops and
369 carbamates. It is noticeable that the Vacaria population owning the lowest specific AChE activity
370 was also the less susceptible to carbaryl. Similar OP resistance associated to reduced AChE's
371 affinity for acetylthiocholine substrate was previously described in *C. pomonella* (Reuveny et al
372 2004). This resistance to chlorpyriphos in OFM populations was also associated with an increase
373 of GST and EST (for both a-NA and p-NA substrates) activities. However, converging to what
374 occurs in *C. pomonella* (Reyes et al, in press), these combined target site modification and
375 detoxification mechanisms do not confer a high level of resistance to the analyzed pesticides. But
376 in the case of these OFM populations, the activity ratios of the detoxifying enzymes were rather
377 low, never exceeding two times more than the reference. The Brazilian populations also slightly
378 differed from the reference strain for their specific AChE activity (0.4- to 0.7-fold) and for
379 AChE's inhibition by carbaryl (1.7- to 2.5-fold in female moths). Comparatively, the 25-fold
380 resistance to carbofuran of Canadian populations of *C. molesta* (Kanga et al. 1997) was
381 associated to a close to 4-fold increase of a-NA and to a 1757- fold decrease of susceptibility of
382 AChE to carbaryl, without any alteration of the MFO and GST activities. Further investigations

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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

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406 populations of *C. molesta* (Siegwart et al 2010a). The sequencing of this gene in *C. moletsa*
407 would thus be of particular interest, to go back to the conserved DNA samples of Brazilian and
408 French populations.
409 The Lages population issuing from the orchard which was the most heavily treated during in the
410 last five years had also the highest GST and EST activities, significantly higher than the Vacaria
411 population, and had also an increased MFO activity when compared to the reference strain. Such
412 activities were not related to an increased tolerance to any of the tested insecticides but
413 conversely to a significantly increased susceptibility to tebufenozide. An increased susceptibility
414 to thiacloprid was also recorded in Lages and Porto populations, without any relation with the
415 observed mechanisms. Regarding the low resistance and activity ratios that were recorded in this
416 study, it may be hypothesized that resistance mechanisms are at the beginning of the selection
417 process, as attested by the large intra population variability and the high activities recorded in
418 few individuals of the population issuing from the most heavily treated orchard in Lages.
419 Moreover, it cannot be excluded that a part of the resistance was lost during the few generations
420 of rearing of the populations in the laboratory without selection pressure, due to the fitness cost
421 usually associated to metabolic resistances (Roush and Plapp 1982, Mc Kenzie and Batterham
422 1991, Boivin et al. 2003) and to the AChE mutations as well (Bourguet et al 2004, Shi et al
423 2004). However this loss of resistance during the rearing process could only lead to an under
424 estimation of the rate of resistant individuals in the analysed populations, without qualitative
425 change in the observed mechanisms.
426 Establishing the baseline susceptibility to insecticides and the baseline activity of enzymatic
427 metabolization systems is required for insecticide resistance monitoring and management (Roush
428 and Tabashnik 1990). This was made possible with this study for insecticide groups which were

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429 the most intensively used in apple orchards during previous decades, i.e. organophosphates,
430 pyrethroids and carbamates. Due to their negative ecological impact, these compounds are now
431 being replaced by more selective or new ones, including insect growth regulators and
432 neonicotinoids. Despite the frequent observation of metabolic resistance to the moulting activator
433 tebufenozide in various lepidopteran species (Sauphanor and Bouvier 1995, Smagghe et al. 1998,
434 Waldstein and Reissig 2000, Cao & Han 2006), the efficacy of this insecticide was conserved
435 against the tested populations. This is also the case for thiacloprid, which is not yet frequently
436 applied in the studied area. The developed and tested method will be used to analyse new
437 populations, trying now to get large collections allowing direct investigations on the F1 neonates
438 of the sampled insects. This method will also be implemented for other insecticides, including
439 the recent compounds that are expected to be registered in Brazil against *C. molesta*.
440

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441

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590 **Table 1: Susceptibility to five insecticides of neonate larvae of one reference strain (S_{it})**

591 **and three Brazilian populations (Vacaria, Porto, Lages) of *C. molesta*.**

Insecticide	Population	N[2]	LC50[3] (95% CL)	LC50 susceptibility Ratio / S_{it}[4]
Chlorpyriphos	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)

592 [1] S_{it} – European reference strain mass-reared on artificial diet since over 10 years.
593 [2] Number of neonates tested.
594 [3] LC_{50} values expressed in mg L^{-1}.
595 [4] Susceptibility Ratio/S_{it}: Resistance ratio = LC50 of Brazilian strain divided by LC50 of the reference strain.
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598 **Table 2: Acetylcholinesterase inhibition by Carbaryl in adults of four populations**

599 **of *C. molesta*.**

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

600 [a] Number of adult month tested.
601 [b] Concentration are expressed in mmol/min/mg of protein for Carbaryl.
602 [c] IC_{50}s estimated by probit analysis; inhibition ratio IR calculated by dividing the IC_{50} for
603 the Brazilian populations (Lages or Porto or Vacaria) by the IC_{50} for our laboratory strain (S_{it}) for each gender.
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Figure Legends
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608 Figure 1: (a) Glutathion S Transferase activity, measured in fluorescence unit formed/min/µg of 609 protein, in reference strains (S_{it}) and Brazilian strains (Vacaria, Porto and Lages) of the oriental 610 fruit moth. (b) Carboxylesterase activity, measured in nmoles of product (α-Naphtol or p-611 Nitrophenol)/min/mg of protein. Letter (Capital one for esterase activity with p-NA and small 612 letter for GST or esterase activity with α-NA) illustrate Tukey test ($p > 0.05$). (c) Acetylcholine 613 esterase activity, measured in nmoles of acetylthiocholine/min/mg of protein, in reference strains 614 (S_{it}) and brazillian strains (Vacaria, Porto and Lages) of the oriental fruit moth. Letter illustrate 615 Tukey test ($p > 0.05$).

616 Figure 2: Distribution of MFO activities (pg of 7-HC/min/abdomen of insect) by populations and 617 sex.

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619 Figure 1

620 **a)**

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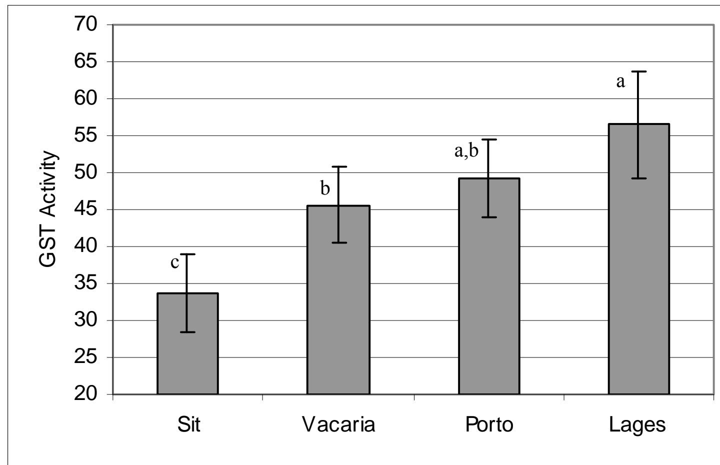

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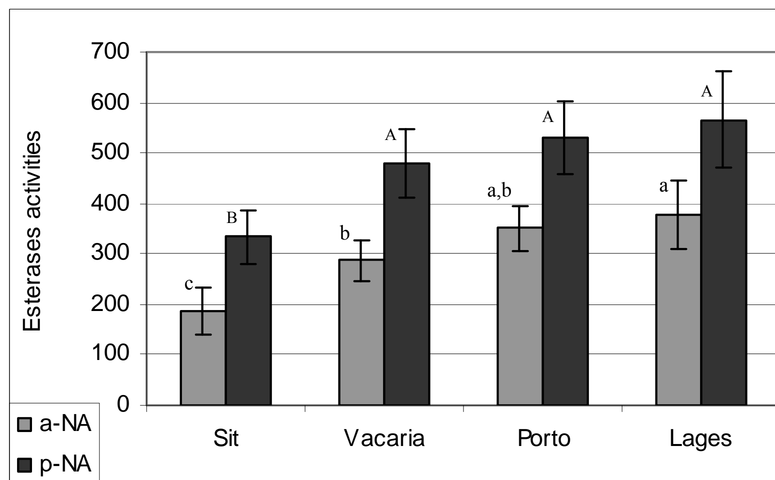
624 **b)**



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629 **c)**

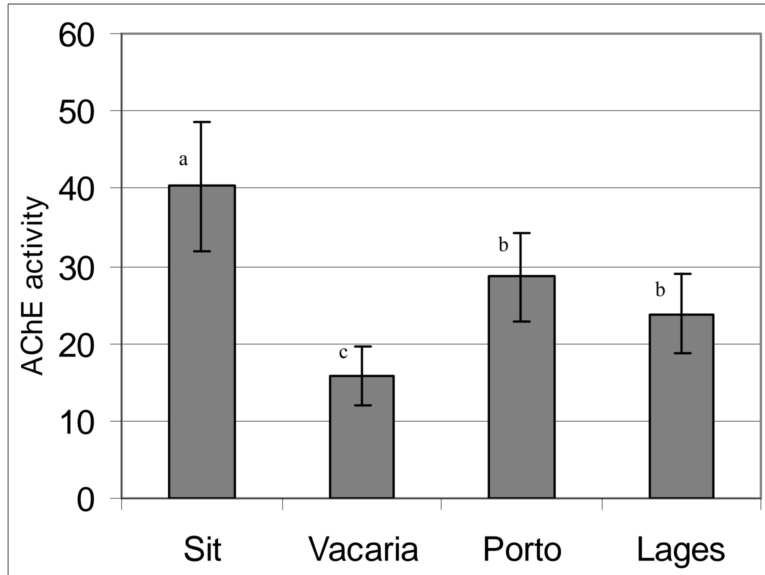

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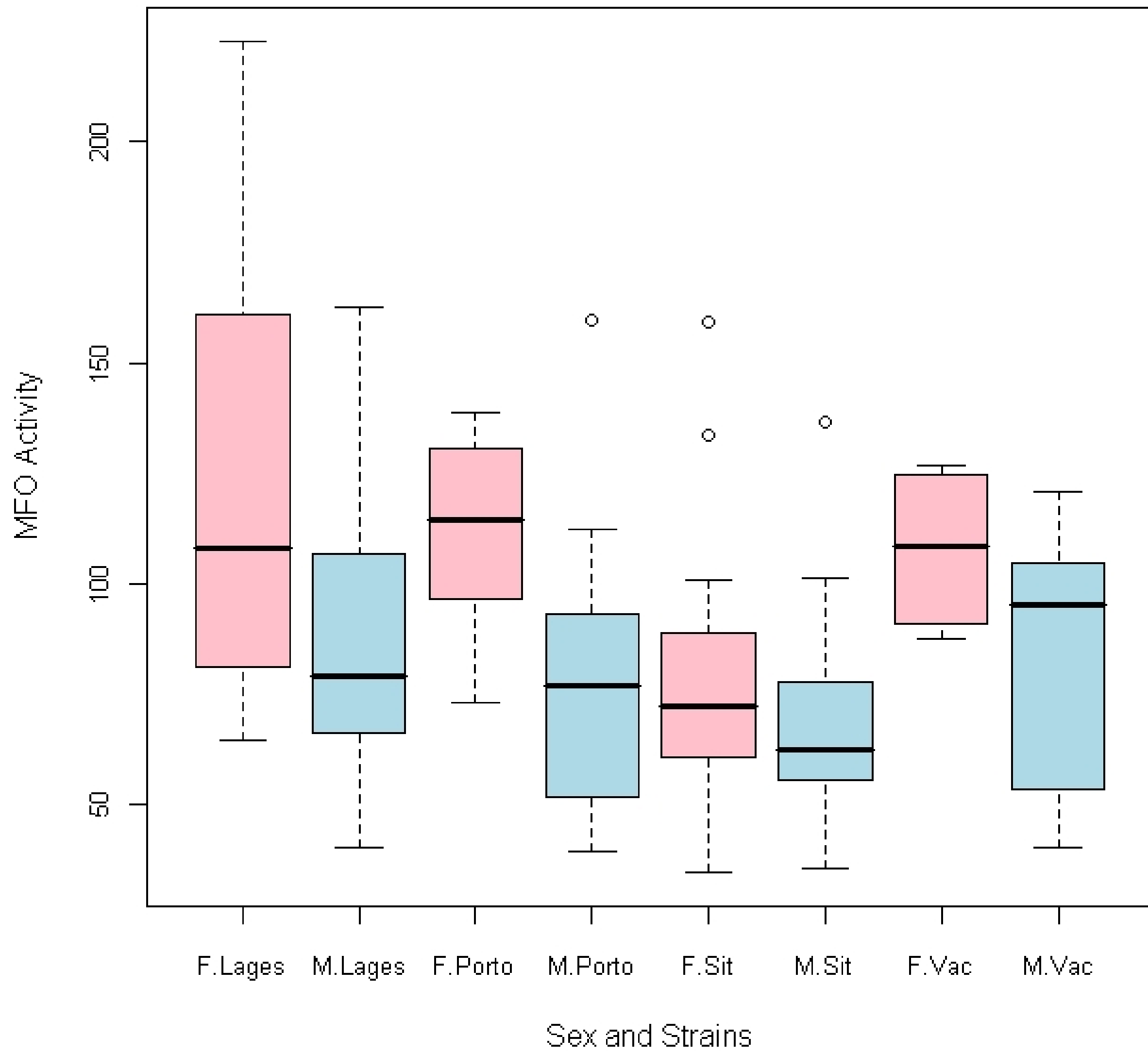
Figure 2



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