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1 **Cross effects of heat stress and three insecticides on the survival**
2 **of the codling moth *Cydia pomonella* (L.): investigating the molecular**
3 **and biochemical mechanisms**

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26 **Abstract**

27 As temperature is expected to strongly increase in the future, understanding temperature-
28 mediated toxicity of insecticides is determinant to assess pest management efficiency in a
29 warming world. Investigating molecular and biochemical mechanisms associated with cross
30 mechanisms of temperature and insecticides on pests' tolerance would also be useful in this
31 context. This study aimed to investigate cross effects between temperature and insecticides
32 on the survival of a major pest, the codling moth *Cydia pomonella*, and their underlying
33 mechanisms. The effect of three insecticidal active ingredients, i.e. chlorantraniliprole,
34 emamectin and spinosad, was assessed at different temperatures on: (i) *C. pomonella*
35 larval survival; (ii) detoxification enzymes activities (cytochrome P450 multi-function
36 oxygenases, carboxylesterases and glutathione S-transferases) and (iii) genes expression
37 of some detoxification enzymes, heat shock proteins and receptors targeted by the
38 insecticides.

39 We observed a decreased efficiency of emamectin and spinosad at high temperature to
40 control the codling moth while no influence of temperature on chlorantraniliprole efficacy was
41 observed. Detoxification enzymes activities were improved by heat stress alone but not by
42 double stress (temperature + insecticides). Moreover, two detoxification genes (*Cyp9A61*
43 and *Gst1*) were over-expressed by a single stress but not by two stresses while *Hsp70* and
44 *Cyp6B2* genes may be involved in tolerance to two stresses in *C. pomonella*. These results
45 confirmed the cross effects of temperature and insecticides on *C. pomonella* for emamectin
46 and spinosad and provided clues to understand how temperature affects the susceptibility of
47 *C. pomonella* to insecticides. They illustrate however the complexity of molecular and
48 biochemical responses of individuals facing multiple stresses.

49

50 Key words: cytochrome P450, carboxylesterases, glutathione S-transferases, heat shock
51 proteins, emamectin, spinosad.

52 **Introduction**

53 Despite their strong negative impact on environment (Stehle & Schulz 2015) and
54 human health (Koureas *et al.* 2012), chemical insecticides are widely employed to
55 manage insect pests. Their efficiency is however known to depend on environmental
56 conditions, including temperature (Matzrafi, 2018) and climate change may thus
57 strongly influence their effectiveness through several mechanisms. First, insecticide
58 concentration in crops and plants is expected to decrease with increasing
59 temperature (Delcour *et al.* 2014) because of increased dilution in plants (Holland &
60 Sinclair, 2004) volatilization (Navarro *et al.* 2007) and biological or chemical
61 degradation in soils (Athanasopoulos *et al.* 2004). Secondly, rising temperature
62 commonly results in faster development, advanced mean emergence date, higher
63 reproductive rate and increased distribution range in insects (Stoeckli *et al.* 2012),
64 resulting consequently in increased pests' abundance and occurrence of outbreaks
65 (Gutierrez *et al.* 2008). Thirdly, the increased number of generations reported with
66 increasing temperature may accelerate appearance of resistance to some
67 insecticides in multivoltine pest species (Maino *et al.* 2018).

68 Such influence of global warming on insecticide use may also result from the
69 temperature- dependency of toxicity that has been reported for a wide range of
70 products (Glunt *et al.* 2014; Saeed *et al.* 2018) although this relationship depends on
71 several factors including the insecticide itself. For example, toxicity commonly
72 increases with temperature for organophosphate insecticides and decreases for
73 pyrethroid insecticides (Satpute *et al.* 2007), but some exception has been reported
74 within several classes, including pyrethroids (Boina *et al.* 2009). Moreover, the
75 relationship between temperature and insecticide toxicity is strongly insect specific,

76 as observed for deltamethrin that is more toxic as temperature increases for the fall
77 armyworm *Spodoptera frugiperda* and the tobacco budworm *Heliothis virescens*, but
78 less toxic for the cabbage looper *Trichoplusia ni* and the boll weevil *Anthonomus*
79 *grandis* (Sparks *et al.* 1982, 1983).

80 Biochemical mechanisms involved in insecticide detoxification or in protection
81 against extreme temperatures have been thoroughly investigated individually, but
82 only few studies focused on those involved in the influence of temperature on
83 insecticides' toxicity. Among potential explanations, increased insecticide toxicity may
84 be due to an increase in locomotor activity and feeding rate occurring at high
85 temperature in insects, therefore increasing the penetration of the product into pest's
86 body (Boina *et al.* 2009; Medrzycki *et al.* 2010). Such mechanism is however unable
87 to explain the negative relationship between temperature and toxicity observed for
88 some products or pest species and other mechanisms should thus be involved.

89 Several enzymes such as glutathione S-transferases (GST), carboxylesterases
90 (CbE) and cytochromes P450 (P450) are known to be involved in insecticide
91 detoxification by organisms and enzymatic activities directly depends on
92 temperature (Okutman Tas & Pavlostathis 2007). They may thus mediate the
93 relationship between temperature and insecticide toxicity. Zhang *et al.* (2015)
94 observed in the diamondback moth *Plutella xylostella* that the activity of these
95 resistance-related enzymes was inhibited at 36 and 40°C compared to 25°C, in
96 conjunction with a reduced resistance to chlorpyrifos. However, changes in activity
97 levels of detoxification enzymes with temperature were not always associated with a
98 change in susceptibility of the mosquito *Aedes aegypti* to four organophosphate
99 insecticides (Polson *et al.* 2012). In *Anopheles arabiensis*, insecticide toxicity

100 decreased with increasing temperature but no changes in the activity of
101 detoxification enzymes occurred (Oliver & Brooke 2017).

102 Heat Shock Proteins (HSPs) may also be involved in the temperature
103 dependency of insecticides toxicity. They are proteins that have been described in
104 most living organisms and are activated by specific genes in response to a wide
105 range of stressful environmental factors to protect organisms (Chapuis *et al.* 2011). It
106 has been proposed that HSP synthesis in response to one stress, temperature here,
107 may increase tolerance to other stresses, including insecticides (Patil *et al.* 1996;
108 Ge *et al.* 2013). For example, increased tolerance to a carbamate and an
109 organophosphate insecticide in two mosquito species may have been induced by
110 exposure to an HSP-inducing temperature (Patil *et al.* 1996; Raghavendra *et al.*
111 2010), although HSPs were not quantified in these studies. Conversely transcript
112 levels encoding *hsp70* were up-regulated in individuals of two pest species exposed
113 to insecticide, *Bemisia tabaci* (Su *et al.* 2017) and *Nilaparvata lugens* (Ge *et al.* 2013),
114 resulting in increased thermotolerance.

115 Detoxification enzymes and HSPs may thus both mediate the influence of
116 temperature on insecticide toxicity and disentangling such mechanisms might help
117 to select or design products adapted to the future climate.

118 The aim of this study was to investigate molecular and biochemical mechanisms
119 involved in cross-effects of temperature and insecticides on mortality of the
120 codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae), a major pest in apple
121 orchards. Three insecticides, commonly used against this species, known to be
122 stable at high temperature (Afssa, 2010; Anses, 2010, 2011) and having different
123 modes of action were chosen: (1) emamectin known to activate glutamate-gated
124 chloride channels (GluCl) specific to invertebrates and also to bind to GABA

125 receptors located at glutaminergic synapses (Wolstenholme 2012; Jansson *et al.*,
126 1997), (2) chlorantraniliprole that inhibits the ryanodine receptor at the level of the
127 muscular fibrils with a myo-blocking effect (Cordova *et al.*, 2006) and (3) spinosad
128 that has a nicotine agonist effect on acetylcholine receptor sites and an antagonist
129 effect on a lower affinity site blocking the same receptors (Salgado, 1998). We
130 determined if the cross-effects between temperature and insecticides (i) had an
131 effect on codling moth larval mortality, providing information on the efficiency of
132 insecticides when temperature increases, and (ii) induced changes in activity and
133 genes expression of detoxification enzymes (cytochromes P450, glutathione S-
134 transferase and carboxylesterases), as well as in gene expression of HSP (*Hsp70*)
135 and receptors targeted by the insecticides (GABA receptor & Ryanodine receptor).

136

137 **Material & Methods**

138 **Insect sample**

139 A *C. pomonella* granulovirus susceptible strain (CpNPP) originating from northern
140 France and reared in laboratory since 1995 was used for this study. In our study,
141 CpNPP was reared on artificial diet at INRAE Avignon (France) without insecticide
142 pressure at 25°C; 16:8 h L:D. After few days of mating, eggs were collected,
143 washed with a water-based solution of dishwashing liquid and bleach and then
144 dried. The neonates emerged are then used for the different experimentations.

145

146 **Bioassays and sample preparation**

147 We tested the influence of both temperature and insecticides on mortality of *C.*
148 *pomonella* larvae. Commercial formulations of Emamectin benzoate: Affirm®
149 0,95%, Spinosad: Sucess 4® 480g/L and Chlorantraniliprole: Coragen® 200 g/l were
150 supplied respectively from Syngenta (France), Dow AgroSciences Distribution
151 (France) and FMC Agricultural solutions (France). We used commercial formulations
152 which are water-soluble to get closer to actual treatments in orchards. For bioassays,
153 96 multiwell plates containing 150µL of artificial diet (Stonefly diet Industries Ltd,
154 Rochester, NY) per well were treated with different concentrations of insecticides
155 diluted in osmosed water (Table 1). A 6µL volume of insecticide solution, or water
156 for control, was deposited per well on diet surface. After 20 minutes of drying,
157 a neonate larva was deposited, and wells were closed with parafilm strips (Reyes
158 *et al.* 2007). The plates were then placed in climatic chambers at four different
159 temperatures: 20°C, 25°C, 30°C or 35°C ($\pm 1^\circ\text{C}$). Mortality rate was assessed at 105-
160 degree days, i.e. 10 days at 20°C, 7 days at 25°C, 5 days at 30°C and 4 days at 35°C.
161 We decided to assess the mortality at different times based on degree days since as

162 temperature increases, as the metabolism accelerates for insects. This results in
 163 shorter development time and increases the need for food. An assessment time based
 164 on degree days allowed us to assess mortality on larvae of the same stage that have
 165 ingested a close amount of insecticide. A larva was considered as dead when not
 166 responding to a probe with dissecting forceps. Missing larvae were subtracted from
 167 the initial number. The corrected mortality was calculated using the Abbott formula,
 168 which corrects insecticide-induced mortality by natural mortality in same bioassay
 169 (Abbott 1925). Each test consisted in 24 individuals per concentration (including
 170 control), per insecticide and per temperature and was replicated at least three times.
 171 To limit experimental variation between bioassays, each replicate started the same
 172 day for the different temperatures.

173 Table 1: Concentrations of insecticide active substance used for bioassays on *C.*
 174 *pomonella* neonate larvae.

Insecticides	Concentrations (mg/L)					
Affirm® (Emamectin)	0.02	0.03	0.05	0.08	0.14	0.22
Coragen® (Chlorantraniliprole)	0.3	0.53	0.95	1.69	3	
Sucess 4® (Spinosad)	0.05	0.15	0.45	1.34	4.01	12

175

176 **Exposure treatments for enzymatic and biomolecular analyses**

177 Exposure treatments were used to quantify the influence of temperature and
 178 insecticide on activity of several detoxification enzymes and molecular analyses.
 179 Codling moth individuals were exposed during their development (from neonatal
 180 larvae to adult emergence) to two temperatures, 20 or 35°C, and to one of the three
 181 insecticides at LC30 concentrations (*i.e.*: estimated concentration, based on probit
 182 regression of bioassay results, resulting in 30% mortality) or water for control

183 individuals (Table 2). Each neonate larva was individually placed in a plastic
184 hemolysis tube containing artificial diet prepared according to Guennelon *et al.* (1981)
185 recipe without formaldehyde. Diet was polluted by dropping 15 µl of insecticide
186 solution on the surface of the diet after its polymerization and dried at room
187 temperature before use.

188

189 Table 2: LC30 (mg/L) of 3 commercial insecticides at 20°C and 35°C on a susceptible
190 *C. pomonella* strain, corresponding to the concentration used for enzymatic and
191 molecular experiments.

Insecticides	LC30	
	at 20°C	at 35°C
Affirm® (Emamectin)	0.04	0.11
Coragen® (Chlorantraniliprole)	0.66	0.39
Sucess 4® (Spinosad)	1.34	2.69

192

193 Adult emergence was checked every day. As soon as adults emerged, organisms
194 were dissected individually to separate thorax from abdomen. The anterior half of
195 the abdomen was immediately used to measure P450 activity. Posterior half abdomen
196 was crushed in 110 µl of HEPES buffer (50 mM, pH 7) on the ice and the obtained
197 homogenate was centrifuged at 15 000 g for 15 min at 4 °C. Supernatant was stored
198 at -80 °C and was later used for CbE and GST measurements the same day (Bouvier
199 *et al.* 2002). This method allowed us to monitor activities of these three enzymatic
200 families on the same individual (Reyes *et al.* 2007; Siegwart *et al.* 2017). We expected
201 to have detoxification enzymes in the fat and digestive tract that are well present in
202 both parts of the abdomen. The anterior part was always kept for P450 determination

203 and the posterior part for GST and CbE determination so that a rigorous comparison
204 could be made between treatments. The thorax was immediately dived in liquid
205 nitrogen and kept at -80°C for molecular analyses.

206

207 **Enzymatic activities**

208 *Cytochromes P450 (P450) activity*

209 The P450 activity was determined using 7-ethoxycoumarin O-deethylation (ECOD)
210 (Ulrich and Weber 1972) adapted for organic tissues analysis in microplates. Each
211 sample was crushed and homogenized with a mini hand-held homogenizer in an
212 incubation solution containing 100 µl of Hepes buffer (50 mM pH 7) with 7-
213 ethoxycoumarin (0.4 mM) on the ice. After a 4h incubation at 30 °C, the reaction was
214 stopped by adding 100 µL of 1.5M glycine buffer (pH 10.3) and the solution was
215 centrifuged at 15 000 g for 2 min at 2 °C. The supernatant was placed in a well
216 of black microplates (96-wells, Corning Costar®, New York, U.S). The 7-
217 hydroxycoumarin (HC) fluorescence was quantified with 380 nm excitation and 465
218 nm emission filters. Three wells per microplates were left without samples but just
219 the mix receiving glycine buffer previous to incubation to have a blank. A ten-fold
220 dilution for males and a five-fold dilution for females was operated before dosage
221 using the Bradford method (Bradford 1976). P450 dosage was performed on
222 this reaction product after fluorescence measurement. The P450 activity was
223 expressed as pg of 7-Hydroxycoumarine (7-HC)/mg of total protein/min by using a
224 standard curve of 7-HC (0.5-4.5 nmoles/well) to convert fluorescence in 7-HC
225 quantity.

226

227 *Carboxylesterases (CbE) activity*

228 Total non-specific CbE activity was measured with α -naphthyl acetate (α -NA) as
229 substrate (Reyes 2007). The reaction mixture was 1 μ l of extract and 194 μ l of 30 μ M
230 α -NA in HEPES buffer (50 mM, pH 7.0) in each microplate well. Three wells per
231 microplate were filled with 1 μ l of HEPES buffer (50 mM, pH 7.0) instead of extract as
232 blank. After 20 min of incubation at 22 °C in darkness, the reaction was stopped
233 and colored by adding 55 μ l of 0.2 % Fast Garnet GBC diluted in 2.5 % sodium
234 dodecyl sulphate solution. Absorbance was recorded at 590 nm, after incubation for
235 20 min in darkness at room temperature. A 10-fold dilution for males and a 5-fold
236 dilution for females was operated before this dosage using the Bradford method
237 (Bradford 1976). The standard curve with α -Naphthol (α -Nol) (0-18 nmoles/well) was
238 elaborated to express CbE activity in nmoles of product/min/mg of total proteins.

239 *Glutathione S-transferase (GST) activity*

240 GST activity was determined in transparent microplates (96-wells, Sterilin®,
241 Newport, UK) using 2,4-dinitro-chlorobenzene (CDNB) as substrate (Nauen *et al.*
242 2002). The reaction mixture consisted of 2 μ l of extract, 198 μ l of a solution
243 containing: 10 μ l of 50 mM glutathione (GSH), 185 μ l of HEPES buffer (50 mM, pH 7.0)
244 and 3 μ L of 50 mM CDNB (Rodríguez *et al.* 2011). Three wells per microplate were
245 filled with 2 μ l of HEPES buffer (50 mM, pH 7.0) instead of extract as blank.
246 Absorbance was measured after 2 min of incubation at 25 °C in kinetic mode every 30
247 seconds at 340nm. We used the molar extinction coefficient (9.6 mM⁻¹.cm⁻¹) of
248 CDNB-glutathione to convert absorbance in μ mol of CDNB-glutathione. The final
249 specific activity was expressed in μ mol of CDNB-glutathione/min/mg of total protein
250 extracted.

251

252 **Molecular analysis**

253 We quantified the influence of temperature and insecticide on expression of genes
254 involved in detoxification processes using the thorax kept at -80°C, as indicated
255 previously. Three replicates of 5 individuals per treatment and per sex were performed.

256

257 *RNA extraction and quality assessment*

258 Total RNA was extracted from frozen samples with the Qiagen RNeasy® Plus
259 Micro Kit (Qiagen). Thorax of 5 individuals were pooled into 350µl of Buffer RLT
260 plus and were grounded using a 3,15 mm steel bead on a 1600 MiniG (Spex®
261 SamplePrep) tissue homogenizer at 1500 strokes/min for 15 seconds. Extraction
262 was then continued as described in the RNeasy® Plus Micro Kit Handbook
263 ("*Purification of Total RNA from Animal and Human Tissue* "; p. 30-38). A DNase
264 treatment was carried out using the RNase-Free DNase set (Qiagen) as described
265 by the manufacturer. Total RNA purity and concentration were evaluated by
266 spectrophotometry using NanoDrop ND-2000 (ThermoFisher) and integrity was
267 verified by 1 % agarose gel electrophoresis.

268

269 *Genes selection and primer design:*

270 The sequences of *C. pomonella* genes involved in response to chemical or
271 thermic stresses were obtained from GenBank (www.ncbi.nlm.nih.gov/genBank) and
272 from the search module available in InsectBase ([http://www.insect-](http://www.insect-genome.com/cydia/)
273 [genome.com/cydia/](http://www.insect-genome.com/cydia/)). According to the literature we have listed 30 genes generally
274 involved in resistance. Among those, we selected the predominant *Cytochrome*
275 *P450* and *Glutathione S-transferase* genes, as they may be involved in xenobiotic

276 detoxification and some resistance mechanisms in *C. pomonella* (Zimmer *et al.* 2018;
277 Wan *et al.* 2019), but also overexpressed after heat shock in some insect species (Liu
278 *et al.* 2017; Yan *et al.* 2013). We also selected *Carboxylesterase* genes that may be
279 involved in insecticide resistance mechanisms in *C. pomonella* (Reyes *et al.* 2007).
280 *Heat shock protein* genes were also selected as they are usually expressed in
281 response to heat stress but could also be overexpressed in response to insecticide
282 treatment (Su *et al.* 2018). Finally, we looked for receptors targeting by the three
283 insecticides we tested, *i.e.* *ryanodine receptor*, *Gaba receptor* and *nicotinic receptors*,
284 that could be specifically overexpressed in response to individual insecticides
285 (Wolstenholme 2012; Jansson *et al.* 1997; Cordova *et al.* 2006; Salgado, 1998).

286 The design of all primers pair was made using the Primer 3 web interface version
287 4-1-0 (<https://bioinfo.ut.ee/primer3/>) (Rozen and Skaletsky 2000), where possible,
288 within 1kb of the 3' end of the genes or of the poly-A tail for complete cDNA synthesis.
289 Selected sequences were manually edited and aligned using Bioedit Software
290 (Bioedit v.7.2.1., Hall, T.A. 1999) to check the specificity of the designed primer
291 between same family genes.

292 Efficiency of all primers pair was assessed by qPCR (see below) against a four
293 points standard curve of serial dilutions (1:5) of cDNA ranging from 2 to 0,016 ng/μl, a
294 melting curve analysis and a 2 % agarose gel electrophoresis were used to check the
295 primers specificity.

296 All primers pair with a unique PCR product and an efficiency ranging from 1.91 to
297 2.11 were kept for gene expression analyses: *Cyp6B6*, *Cyp6B2*, *Cyp6B1*,
298 *Cyp9A61*, *Gst1*, *GstD1*, *GABAreceptor*, *Hsp70*, *Ryanodinereceptor* and *Actin*, as the
299 housekeeping gene (supplementary data Table S1). *Carboxylesterases* and *nicotinic*
300 *receptors* genes had no sufficient efficiency to be kept for gene expression analyses.

301 *Gene expression analyses:*

302 The gene expression was assessed by Reverse transcription followed by quantitative
303 PCR. Total RNA (200ng) was used for first strand cDNA synthesis, using oligo-(dT)15
304 primers and the Reverse Transcriptase Core Kit (Eurogentec, Liège, Belgium)
305 according to the manufacturer's instructions. qPCR were performed in a 384-well
306 plate using a CFX384 cycler (BioRad, Hercules, CA, USA). qPCR amplification was
307 performed using the Takyon No Rox SYBR Core kit dTTP blue (Eurogentec, Liège,
308 Belgium) in a 10 µl final volume containing 20 ng of cDNA and 250 nM of forward
309 and reverse primers. The amplification program used was: 4 min at 95°C, 40 cycles
310 of 10 sec at 95°C, and 1 min at 60°C. For amplicon specificity confirmation, each
311 qPCR program was followed by a melting curve assay by increasing temperatures
312 from 60 to 95°C, with 5s and each 0.5°C steps. Each biological condition was triplicate
313 and each reaction was carried out in an independent technical triplicate and
314 included a no-template control, a reagent-only control and a no-reverse-transcriptase
315 control.

316 Quantitation cycles (Cq) were defined based on the mean of the three technical
317 replicates and then averaged within biological replicates. The geometric mean of the
318 expression level of actin (ACT) was used as an internal control (reference) to
319 normalize the expression data for the target genes.

320 The relative expression levels of target genes compared with reference gene were
321 calculated as fold changes of the expression using the $2^{-\Delta\Delta Cq}$ method:

$$322 \quad 2^{[(Cq_{(target, sample)} - Cq_{(reference, sample)}) - (Mean(Cq_{(target, calibrator)} - Cq_{(reference, calibrator)})]}$$

323 (Livak and Schmittgen, 2001),

324 where the mean Cq values corresponded to the mean of the three biological
325 replicates. To assess the effect of growth temperature and insecticide, the gene

326 expression of each treatment was related to gene expressions of control individuals
327 grown at 20°C. Moreover, sexes were analyzed separately to avoid differential
328 expression between gender.

329

330 **Data analyses**

331 In order to calculate the different LC50 for each temperature and each insecticide,
332 dose-response curves were fitted with the package drc using R studio software (Ritz
333 *et al.* 2015; Rstudio team 2020 version 1.3.1073). The function drm of this package
334 was used to calculate the different LC50 presented in results and the function
335 EDcomp of this package has been used to compare each concentration and
336 temperature tested.

337 For enzymatic activities, differences between insecticide treatments, temperatures and
338 sexes were analysed using a Kruskal-Wallis test followed by a Wilcoxon rank-sum
339 test performed using R software.

340 For gene expression analysis, the data presented are the averages of three
341 biological replicates (each in technical triplicates). Glm gaussian family tests were
342 used to test for differences in gene expression with temperature and insecticide as
343 factors.

344

345

346

347

348

349

350 **Results**

351 **Effect of temperature on *C. pomonella* susceptibility to insecticides**

352 Temperature had a significant impact on *C. pomonella* LC50 for emamectin with
 353 respectively twice and thrice the concentration of chemical needed to kill 50% of the
 354 larvae at 30°C (p=0.008) and 35°C (p =0.009) compared to 20°C (Table 3).
 355 Temperature also had a significant impact on LC50 for Spinosad with fourth the
 356 concentration of chemical required to kill 50% of the larvae at 25°C, 30°C and 35°C
 357 compared to 20°C (p = 0.04) (Table 3) while no significant difference was observed
 358 between the three highest temperatures. Temperature did not significantly
 359 influence the mortality of larvae exposed to chlorantraniliprole (Table 3).

360 Table 3: LC50 measured for three insecticides used against larvae of a *C.*
 361 *pomonella* susceptible strain CpNPP at four different temperatures. Different letters
 362 indicate significant differences between treatments. (EDcomp, α=0.05).

Temperature	20°C		25°C		30°C		35°C	
	n	LC ₅₀ (confidence interval)	n	LC ₅₀ (confidence interval)	n	LC ₅₀ (confidence interval)	n	LC ₅₀ (confidence interval)
Affirm® (Emamectin)	1427	0.069 a (0.05-0.09)	1491	0.106 b (0.09-0.12)	497	0.134 c (0.11-0.16)	836	0.186 d (0.15-0.22)
Coragen® (Chlorantraniliprole)	431	1.052 (0.8-1.3)	572	1.049 (0.77-1.32)	430	0.907 (0.65-1.32)	427	0.703 (0.49-0.91)
Sucess 4® (Spinosad)	1001	0.792 a (0.31-1.27)	1172	4.583 b (3.25-5.91)	1328	3.617 b (2.38-4.85)	1144	4.737 b (3.45-6.02)

363

364 **Effect of temperature and insecticide on *C. pomonella* enzymatic activities**

365 We did not observe any influence of the sex on enzymatic activities. Both sexes
366 were thus pooled in analyses.

367 **P450 activity** was higher in controls and chlorantraniliprole-exposed individuals
368 that developed at 35°C (average, *i.e.* mean, comprise between 41.2 and 50.0 pg of
369 7EC.min⁻¹.mg protein⁻¹, n=123, n=61) compared to 20°C (average comprise between
370 39.6 and 36.9 pg of 7EC.min⁻¹.mg protein⁻¹, p=0.0404, n=164, n=36). Temperature
371 did not influence P450 activity when individuals were exposed either to spinosad
372 (n=50, n=44) or emamectin (n=45, n=49) (Fig. 1A). Regarding the insecticide effect
373 at 20°C, P450 activity was not modified either after spinosad or emamectin
374 exposure, while a significant decrease was observed after chlorantraniliprole
375 exposure (p<0.01).

376 Higher temperature resulted in higher **CbE activity** for control (n=144, 20°C) and
377 chlorantraniliprole-exposed individuals (35°C n=69, 20°C n=38) but not for those
378 exposed to spinosad (n=51, n=52) or emamectin (n=50, n=53, control 20°C
379 n=103). The highest carboxylesterases activity was recorded in individuals that
380 developed at 35°C without insecticide (average 87.9 nmol anaphtol.min⁻¹.mg protein⁻¹
381 p<0.001) while the lowest activity was observed in individuals that developed
382 at 20°C and were exposed to chlorantraniliprole (average 78.6 nmol anaphtol.min⁻¹
383 .mg protein⁻¹ p<0.001) (Fig. 1A). No significant difference was observed between
384 other treatments.

385 **GST activity** was significantly higher in control individuals that developed at
386 35°C (average 638.8 nmoles of CDNB-GSH conjugate.min⁻¹.mg protein⁻¹
387 p=0.0298, n=144) compared to 20°C (n=143). Temperature did not influence GST
388 activity when any of the three insecticides was added to the diet. No significant

389 difference was detected after insecticide exposure compared to the control. Strong
390 variability between individuals was observed for this enzyme (comprised between
391 103 and 1867 nmoles of CDNB-GSH.min⁻¹.mg protein⁻¹, chlorantraniliprole n=38,
392 n=69; spinosad n=51, n=52; emamectin n=50, n=53) (Fig. 1B).

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394 **Effect of temperature and insecticide on *C. pomonella* candidate genes** 395 **expression**

396 **Cyp.** Temperature and insecticides did not significantly influence the relative
397 expression of *Cyp6B1* or *Cyp6B6* although females exposed to spinosad or
398 emamectin tended to over- express *Cyp6B1* gene in comparison with control
399 females. We observed a significant 7-fold higher expression of *Cyp6B2* in females
400 exposed to both high temperature and emamectin in comparison to control females
401 (*glm*, *p*=0,0462). No other significant difference was detected between treatments
402 although a higher temperature resulted respectively in a 5- and 4-fold increase in
403 *Cyp6B2* relative gene expression in 35°C-control or 35°C- chlorantraniliprole exposed
404 males (Fig.2, Supplementary figure S2). A significant 6-fold over-expression of
405 *Cyp9A61* gene was observed in males exposed to 35°C in the absence of
406 insecticide compared to 20°C-control individuals (*glm*, *p*=0,0064). No other
407 difference between treatments was detected (Fig.2).

408 **GABA receptor.** Temperature and insecticides did not significantly influence the
409 relative expression of *GABA*receptor (Fig.2).

410 **Gst.** In absence of insecticide, exposure to a higher temperature resulted in a
411 significant 8-fold over-expression of *Gst1* gene in males (*glm*, *p*=0,0017) and a 3-
412 fold non-significant over-expression in females. No other significant difference was
413 observed although a 4-fold increase in expression was noted for males exposed

414 to both high temperature and emamectin in comparison with control males and
415 a 3-fold over-expression was noted for females exposed to both high temperature
416 and chlorantraniliprole in comparison with control females (Fig.2, Supplementary
417 figure S2). Neither temperature or insecticides influenced the relative expression of
418 *GstD1* (Fig.2).

419 **Hsp.** Exposure to 35°C induced a strong over-expression of *Hsp70* gene in both
420 sexes compared to 20°C (Fig.2, Supplementary figure S2), whether insecticides were
421 applied or not. Insecticides did not influence HSP gene expression when temperature
422 was kept at 20°C.

423 **Ryanodine receptor.** Heat exposure resulted in a significant 2-fold increase in
424 *Ryanodine receptor* gene expression in males compared with lower temperature
425 when no insecticides were applied (*glm*, $p=0,0419$) while no other significant
426 difference was detected between treatments (Fig.2).

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436 **Discussion**

437 Understanding mechanisms involved in the temperature-dependency of insecticides
438 toxicity is determinant to assess and improve their efficiency in a warming world. An
439 increase in temperature decreased the toxicity of emamectin and spinosad on our
440 strain of *C. pomonella* while it did not influence the toxicity of chlorantraniliprole.

441 Such influence may be partly explained by changes in activity and gene expression
442 of detoxification enzymes and *Hsp70*.

443 The negative relationship that we observed between temperature and Spinosad
444 toxicity on *C. pomonella* is consistent with a study by Mansoor *et al.* (2014) who
445 reported a decrease in the toxicity of this insecticidal active ingredient from 20°C to
446 32°C in the cotton mealybug *Phenacoccus solenopsis*. In contrast to our results
447 however, toxicity of emamectin was previously reported to increase by 1000-fold from
448 16°C to 31°C in the diamondback moth *P. xylostella* (Li *et al.* 2004) and by a 1.64-
449 fold from 20°C to 32°C in *P. solenopsis*. Similarly, a positive relationship
450 between temperature and chlorantraniliprole toxicity was reported in *P. xylostella*
451 (Teja *et al.* 2018) while we did not observe any influence of temperature on the toxicity
452 of this insecticide on the codling moth.

453 Such differences between species in the temperature-dependency of an insecticide
454 toxicity are well-known (Sparks *et al.* 1982, 1983) but to our knowledge, no
455 hypothesis was previously proposed to explain this interspecific variation. First, the
456 efficiency of biochemical pathways involved in insecticide detoxification may vary
457 between species and with temperature. Secondly, differences in the experimental
458 procedures may induce important differences between studies. Most experiments
459 focused on transient 48h stress effects of temperature and insecticides on mortality

460 (e.g. Boina *et al.* 2009). In our study, we exposed *C. pomonella* to those stresses
461 during the complete development of codling moth larvae, which is consistent with
462 what may happen with climate change in agroecosystems since global warming
463 would be effective during the whole life cycle of pests. Recently, Oliver & Brooke
464 (2017) reported a lower mortality in *Anopheles arabiensis* individuals either exposed
465 to deltamethrin and 1-cyhalothrin after a transient 3h heat shock. However, this
466 lower mortality was only temporary as the mortality increased again within few hours
467 for deltamethrin. Medrzycki *et al.* (2010) observed that developmental temperature
468 differently influenced the toxicity of dimethoate in *Apis mellifera* according to their
469 age. Adult workers emerging at 33°C were indeed more susceptible to this insecticide
470 than those emerging at 35°C while the opposite pattern was observed in larvae. These
471 two studies highlight the importance of the timing and duration of thermal exposure on
472 subsequent susceptibility to insecticide intoxication. Temperature is known to mediate
473 enzyme activities and gene expression in insects (Swain *et al.* 2009; Yang *et al.*
474 2016a). We observed in *C. pomonella* that a high developmental temperature
475 induced higher CbE, P450 and GST activities. Such positive relationship between
476 temperature and activity of defensive enzymes is consistent with previous studies on
477 other insect species. High temperatures indeed resulted in increased activity of
478 CbE in the mosquito *Culex quinquefasciatus* (Swain *et al.* 2009) or GST in the
479 silkworm *Antheraea mylitta* (Jena *et al.* 2013). In codling moth, insecticide
480 resistance mechanisms are well known and GST, EST and P450 are major enzymes
481 involved in detoxification (Reyes *et al.* 2007). The simultaneous increase in
482 tolerance to spinosad and emamectin and in the activity of these three enzyme
483 families with temperatures thus suggests that these two observations may be
484 correlated. Changes in insecticide toxicity with temperature were also correlated

485 with changes in activity of the same detoxification enzymes in the pest *P. xylostella*
486 (Zhang *et al.* 2015). Such correlations were however not found in two mosquito
487 species. In *A. arabiensis*, insecticide toxicity decreased with increasing
488 temperature but no changes in the activity of detoxification enzymes occurred (Oliver
489 & Brooke 2017). In *Aedes aegypti*, some changes in activity levels of detoxification
490 enzymes with temperature were not associated with a change in susceptibility to
491 insecticides (Polson *et al.* 2012). We observed close results for codling moth
492 tolerance to chlorantraniliprole that did not vary with temperature despite the
493 increase in activity of detoxification enzymes. Such difference with results on
494 emamectin and spinosad may be explained by the non-involvement of those
495 enzymes in chlorantraniliprole tolerance reported recently for this pest in Spain
496 (Bosch *et al.* 2018), and contrarily to observations on other lepidopterans (Mao *et al.*
497 2019; Yin *et al.* 2021). These differences underline that stress responses may vary
498 according to the insect species studied but also according to intrinsic properties of
499 the insecticidal active substances and may explain differences observed between
500 studies.

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502 Additionally to the increase in enzymes activity, we observed that genes of at least
503 two detoxification enzymes (*Cyp9A61* and *Gst1*) were over-expressed in males in the
504 presence of heat stress, suggesting that they may be involved in increased tolerance
505 to emamectin and spinosad at high temperature. These genes are indeed commonly
506 associated with chemical detoxification (Zimmer *et al.* 2018). In particular, some
507 genes encoding GSTs have been reported to be involved in the detoxification of
508 lambda-cyhalothrin in *C. pomonella* (Hu *et al.* 2019, 2020; Wang *et al.* 2019; Wei *et al.*
509 2020; Liu *et al.* 2014). The *GSTd1* gene is induced after exposure to methomyl,

510 carbaryl, deltamethrin and lambda-cyhalothrin in *C. pomonella* (Yang *et al.* 2016b, Liu
511 *et al.* 2014). Similarly, some CYP450-encoding genes are involved in insecticide
512 detoxification in *C. pomonella*. The *Cyp9A61* gene is induced after exposure to
513 chlorpyrifos-ethyl, lambda-cyhalothrin, deltamethrin, methomyl, carbaryl and
514 imidacloprid in *C. pomonella* (Yang *et al.* 2013, 2016b). The involvement of these
515 genes in the detoxification of different insecticides in *C. pomonella* is well known and
516 was recently compiled in a literature review by Ju *et al.* 2021. Liu *et al.* (2017)
517 similarly reported that a 40°C heat shock for 4h upregulated genes encoding
518 glutathione S-transferase as well as Cyp450 genes from CYP3, CYP6 and CYP9
519 clades in the moth *Glyphodes pyloalis* while a Delta class glutathione S-transferase
520 gene was upregulated in response to heat stress in *Apis cerena cerena* (Yan *et al.*
521 2013). We also observed significant differences in overexpression of certain genes
522 coding for particular detoxification enzymes between males and females (*i.e.* *Cyp6B2*,
523 *Cyp9A51*, *Gst1*) while enzyme activities did not vary with gender. Enzymatic activities
524 reflect the activity of an enzymes' family while gene expression focuses on a single
525 enzyme. We may expect that different enzymes were activated between the two sexes,
526 resulting in a similar global activity for an enzymes' family despite differences in gene
527 expression of a single enzyme. We did not observe any change in expression of
528 other genes coding for detoxification enzymes or molecular targets of insecticidal
529 active substance, except a slight over-expression of the gene coding for the
530 ryanodine receptor, the target of chlorantraniliprole, at 35°C. This change was
531 however not associated with a change in the susceptibility to this insecticide for *C.*
532 *pomonella*.

533 As for *Cyp9A61* and *Gst1*, *Hsp70* gene was upregulated in response to high
534 temperature. Heat Shock Proteins have a primary function as molecular chaperones,

535 promoting correct refolding and preventing aggregation of denatured proteins
536 (Johnson *et al.* 1998), and their upregulation in response to heat has been commonly
537 reported (Sorensen *et al.* 2003). Some previous studies also reported HSP
538 upregulation in response to insecticides (Patil *et al.* 1996), resulting in increased
539 thermotolerance of pest species (Ge *et al.* 2013; Su *et al.* 2018). In our study,
540 insecticide exposure did not influence *Hsp70* expression for individuals that
541 developed at 20°C. Drawing conclusion on the role of *Hsp70* in insecticide tolerance
542 is thus uncertain for *C. pomonella* considering our results.

543 Surprisingly, the increase in GST, CbE and P450 activity and in expression of
544 *Cyp9A61* and *Gst1* genes that we observed at high temperature for control
545 individuals disappeared when emamectin or spinosad were applied. One exception
546 to this apparent absence of response to double stress was the *Cyp6B2* gene from
547 CYP6 clade that was significantly overexpressed in 35°C emamectin-treated females
548 compared to females grown at 20°C. This gene, which is involved in detoxification of
549 deltamethine and azinphos methyl in *C. pomonella* (Wan *et al.* 2019), may also have
550 a strong effect on the detoxification of emamectin and its over-expression may be
551 maintained despite the double stress.

552 Several hypotheses may be proposed to explain the apparent absence of
553 biochemical or molecular responses to two stresses (heat + insecticide) in
554 comparison to one stress. The mobilization of the enzymes on the insecticidal
555 substrates would make them less accessible for the substrates proposed for the
556 analysis. Indeed, insecticides can compete with the substrate for these enzymes
557 when the active site is the same for the two molecules and this phenomenon
558 increases when the enzymes simply do molecular sequestration and remain bounded
559 to the insecticide for a longer time (Kostaropoulos *et al.* 2001; Goh *et al.* 1995;

560 Despres *et al.* 2007). We may also hypothesize that energy may be preferentially
561 allocated to the expression of genes regulating more ubiquitous mechanisms, such
562 as HSP (Sorensen *et al.* 2003), to protect individuals against multiple stresses.
563 *Hsp70* gene was indeed still upregulated when two stresses were applied although
564 this upregulation was less pronounced when individuals faced double stress
565 compared to heat stress alone. Increasing the expression of *Hsp70* gene is
566 associated with increased energy allocation to transcription, translation and protein
567 function (Hoekstra & Montooth 2013; Liu *et al.* 2008). We may expect that individuals
568 facing multiple stresses would reduce the costly expression of HSP compared to
569 individuals facing one stress to cope with other biochemical and molecular responses
570 involved in stress responses.

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577 **Conclusion**

578 Our results highlight the cross-effects of temperature and insecticides on *C.*
579 *pomonella* mortality for emamectin and spinosad and provide important clues for
580 understanding how temperature affects *C. pomonella* susceptibility to insecticides.
581 *Hsp70* may play a role in dual stress (heat+insecticide) responses, and *Cyp6B2* may
582 also be involved in mechanisms of reduced sensitivity to emamectin and high
583 temperatures. If one stress induces overexpression of detoxification genes, two do
584 not. Allocation of cellular energy to more ubiquitous multi-stress tolerance pathways
585 seems likely. We would need to move to more comprehensive RNA seq gene
586 expression techniques to get a more complete picture of the selected defense
587 pathways based on the number and types of stresses. Our observations echo notions
588 of evolutionary biology that define "landscape fitness" in which individuals subjected
589 to environmental changes will be selected on different traits able to respond to
590 stresses without affecting too much its other vital functions, to move towards the best
591 evolutionary compromise (Yang *et al.* 2021).

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600 **Acknowledgments**

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602 pugère” for the financial support of M. Perrin doctoral fellowship.

603 **Figure captions**

604 Fig. 1: Activity of Relative cytochromes P450 (pg 7EC.min-1.mg protein-1) (**A**),
605 Carboxylesterases (nmol anaphtol.min-1.mg protein-1) (**B**), and Gluthatione S-
606 tranferase (CDNB-GST conjugate.min-1.mg protein-1) (**C**) measured in *C. pomonella*
607 adults emerging from substrate treated with LC30 concentrations of three insecticides
608 and exposed to two different temperature. Bars with different letters are significantly
609 different (wilcoxon test, $\alpha = 0.05$).

610 Fig. 2: Heatmap showing fold-change values calculated using the comparative Ct
611 method for males (**A**) and females (**B**) using control 20°C individuals as reference
612 (see section “Materials and Methods” for details). Variations in green colors indicate
613 an increased (darkest) or a decreased (lighter) genes expression for 35°C-treated
614 individuals compared to 20°C-control individuals. Significant treatment effects are
615 noted with * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) (glm, $\alpha = 0.05$). (Color
616 reproduction web and print).

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890 **Supplementary data Table S1**

891 All primers were designed specifically for this study except Hsp70-2 which are from Yang
 892 *et al.*
 893 2016.

Gene ID	Name	Forward primer 5'--> 3'	Reverse primer 5'-->3'
CPOM03546	CYP 6B6	ACTGGTAGGTGCAATTTTAAGGG	CTAGCAACCTCTGGAGTTCCG
KC832920	CYP 9A61	CAAATACCGCTCTGGGCATAC	GATACGCCATCACCTTTACTTCG
CPOM05212	CYP 6B2	ATCCTTTTCGTGGTTATCAGTGAG	CCCTCCGATTAGCTTGTTGAA
CPOM08435	CYP 6B1	ACTACCCGAACCCTGATGTC	TGAAGGCTCCACCCTGTAG
CPOM06919	GST 1	GATCAATCCACAACATACGGTTCC	GGTGGGAACAGAATGCTACAATC
CPOM00366	GST D1	CACCACGGAGAACAGCTCAAG	CTGCCCTTGCCATATTTGTTG
CPOM16859	GABA receptor alpha-6	CGTGCATCCTCATCGTAGTCC	GTAATGGACGCCAGCAAATTC
CPOM13918	Ryanodine receptor	GATGAGGTCAACAGGAATTGTCAC	TGATGGCGAGCAGGATAATG
JN863695	HSP70-2	GCCAACGACCAGGGTAACAG	CGACCTGTATTTTGGGCTTG
KC832921	Beta-actine	CGAGGCTCTTTTCCAACCC	AGGCGAGGATCGACCCG

894 Gene ID correspond to the Genbank accession number or InsectBase nomenclature.

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905 **Supplementary figure S2:** Relative gene expression (\pm SEM) of Cyp6B2 (A), Gst1 (B),
 906 and Hsp70 (C) in *C. pomonella* emerging adults from substrate treated with LC30
 907 concentrations of emamectin, chlorantraniliprole (Chlo) and spinosad at an
 908 optimum temperature (20°C) and an extreme temperature (35°C) compared to control
 909 without insecticide at 20°C. Significant differences between treatment effects are
 910 noted with * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$), glm, $\alpha = 0.05$.

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