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1 **Effects of bisphenol A on post-embryonic development of the cotton pest *Spodoptera***  
2 ***littoralis***

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14 A.M., A.M.C., I.B., V.B., C.B. and C.B. carried out the experiments. M.D. helped in the statistical  
15 analysis. D.S. conceived the original idea, supervised the project and wrote the manuscript with  
16 support from T.C., M.M. and C.B. All authors provided critical feedback and helped shape the  
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25  
26 **Abstract:**

27 Endocrine-disrupting chemicals encompass a variety of chemicals that may interfere with the  
28 endocrine system and produce negative effects on organisms. Among them, bisphenol A is  
29 considered a major pollutant in numerous countries. The harmful effects of BPA on environmental  
30 and human health are intensely studied. However, the effects of BPA on terrestrial insects are still  
31 poorly investigated, despite that several plants can accumulate BPA in their tissues, leading to  
32 potential contamination of herbivorous insects. Here, we used the leafworm *Spodoptera littoralis*, a  
33 polyphagous species, to study BPA effects on post-embryonic development. We studied the effects  
34 of BPA ingestion at environmental doses (e.g., 0.01, 0.1, and 1 µg/g of BPA) and high doses (e.g.,  
35 25 µg/g) on larval weight and stage duration, pupal length and sex ratio. BPA effects were  
36 investigated in more detail during the last larval instar, a crucial period for preparing pupation and  
37 metamorphosis, which are under endocrine control. We monitored the haemolymph concentration  
38 of ecdysteroids, hormones controlling moult and metamorphosis, as well as the expression levels of  
39 several nuclear receptors involved in the ecdysteroid signalling pathway. Our integrative study

40 showed that, upon exposure doses, BPA can induce various effects on the viability, developmental  
41 time, growth and sex ratio. These effects were correlated with a delay of the ecdysteroid peak during  
42 the last larval instar and a modification of expression of EcR, USP, E75AB, E75D and Br-c. We  
43 provide new evidence about the events that occur after BPA exposure in insect contaminated by  
44 food ingestion.

45

46 **Key words:** Insect crop pest, Bisphenol A, Post-embryonic development, molecular expression,  
47 Spodoptera, ecdysteroid

48

## 49 1. Introduction

50 Endocrine-disrupting chemicals include a variety of chemical compounds that may interfere  
51 with the endocrine system of organisms, their descendants, or (sub) populations. Bisphenol A (BPA,  
52 CAS No. 80-05-7), a chemical used in various plastic consumer products, is now considered a major  
53 pollutant in several countries (Andrady & Neal 2009, Bauer & Herrmann 1997; Staples et al., 2002).  
54 BPA is currently included in the candidate list of substances of very high concern for authorization,  
55 as in the REACH Regulation. BPA is mostly resistant to environmental degradation (half-life from 1  
56 day to 12 months in water and soil) (Howard, 1989) and can be widely dispersed in the environment  
57 through different ways, such as sewerage systems, waste disposal and spraying of contaminated  
58 water (Fromme et al., 2002; Porter & Janz, 2003; Wang et al., 2011; Casals-Casas & Desvergne,  
59 2011). Little is known about BPA atmospheric distribution and transport; however, different ranges  
60 of concentrations were measured in the atmospheric aerosols from urban, rural, marine and polar  
61 regions from 1 to 17400 pg/m<sup>3</sup> (Fu & Kawamura, 2010; Salapasidou et al., 2011).

62 The disruption mechanisms of EDC on steroid signalling pathways are increasingly well  
63 understood (Craig et al., 2011). BPA effects on human and vertebrate health are now well  
64 documented (Siracusa et al. 2018; ANSES, 2018; Oehlmann & Schulte-Oehlmann, 2003). As an  
65 EDC, BPA can disturb the production, release, metabolism, and elimination of hormones. This  
66 chemical can also interfere with hormone-receptor interactions and block the accessibility of a  
67 receptor to its natural ligand. Direct binding on hormone receptors and inappropriate activation of  
68 steroid signalling pathways have also been reported. Several harmful effects on reproduction, growth  
69 and development of BPA in various organisms, including humans, have been described (Wetherill  
70 et al., 2007; Vanderberg et al., 2012; Stoker et al., 2008).

71 The effects of EDCs, especially BPA, on invertebrates (95% of known animal species) are  
72 still poorly studied, despite their tremendous ecological importance and predominance in food webs  
73 (deFur et al., 1999; Oehlmann & Schulte-Oehlmann, 2003). Moreover, few investigations have been  
74 conducted on terrestrial species (Babic et al. 2016; Michail et al., 2012). Previous studies mainly  
75 focussed on aquatic insects, such as *Chironomus* larvae, and have shown an effect of BPA on  
76 moulting, growth and development (Lee & Choi, 2007; Watts et al., 2001). As in vertebrates, all the  
77 processes under endocrine control in insects, such as development and reproduction, could indeed  
78 be disrupted by EDCs (Rodríguez et al., 2007; Zou, 2005). Inappropriate inductions of the steroid

79 signalling pathway, involving the nuclear receptor ecdysone receptor (EcR) and its heterodimeric  
80 partner, Ultraspiracle (USP), have been reported in several aquatic arthropods exposed to BPA  
81 (Planelló et al., 2011; Tarrant et al., 2011; Hanioka et al., 2008).

82 The presence of EDCs in the environment leads to the exposure of terrestrial organisms by  
83 ingestion of contaminated water or food, or direct exposure through external epithelia (Bar-EI &  
84 Reifen, 2010; Kaddar et al., 2008; Zalko et al., 2011; Watanabe et al., 2005, Lenoir et al., 2016,  
85 Cuvillier –Hot et al., 2014). In the housefly *Musca domestica*, exposure to environmentally relevant  
86 concentrations of BPA (e.g., 100 to 100 µg of BPA per kg of artificial diet) during the early stages of  
87 the life cycle resulted in various disorders in pupal weight and sex ratio (Izumi et al., 2008). In  
88 *Drosophila melanogaster*, opposite results were observed depending upon the strain used: ingestion  
89 of food contaminated with environmental concentrations of BPA (e.g., 8.8 µg/L) in an ebony mutant  
90 strain (Quesada-Calderón et al. 2017) had no effect on the components of fitness (developmental  
91 time and fertility), whereas using a wild-type strain demonstrated effects of BPA from 0.1 mg/L to 10  
92 mg/L on development and growth. In Weiner et al. (2014), a dose of 0.1 mg/L of BPA mixed with  
93 food increased larval growth coupled with an earlier onset of pupation in *D. melanogaster*. This  
94 treatment also increased lipid content and disrupted growth by inhibiting the expression of insulin-  
95 like peptides (Williams et al., 2014).

96 Interestingly, some studies have focused on the effects of BPA on a crop pest, the moth  
97 *Sesamia nonagrioides* (Michail et al., 2012; Kontogiannatos et al., 2015), a species considered an  
98 important pest of sorghum and corn, and have shown that these insects can be contaminated by  
99 BPA polluting water from rivers, reclaimed irrigation water or precipitation (Ferrey et al., 2018). High  
100 BPA levels were measured after root or foliar exposure in tomato fruit from 18 to 27 µg/kg and in  
101 lettuce leaves from 80 to 129 µg/kg (Lu et al., 2013, 2015; Mijangos et al., 2015). Ferrara et al (2006)  
102 also measured BPA in roots and shoots of broad bean and tomato after 21 days of growth, indicating  
103 that bioaccumulation of BPA occurred in these two vegetables. Contamination of *S. nonagrioides*  
104 larvae by ingestion of artificial diets containing lower doses of BPA (1-10 µg/L) induced a decrease  
105 in larval weight, whereas higher doses (100 µg/L, 1 mg/L and 10 mg/L) induced an increase in weight  
106 or various abnormal phenotypes during the last larval and pupal stages (Michail et al., 2012;  
107 Kontogiannatos et al., 2015). Such moulting abnormalities during larval development and  
108 metamorphosis were also reported by Kontogiannatos et al. (2015), together with an increased time  
109 for the transition between larval and pupal instars in *S. nonagrioides*. Studies on cell lines from *D.*  
110 *melanogaster* or *Bombyx mori* suggested that BPA could be able to bind to the EcR/USP complex  
111 that induces the genetic cascade of responses to 20-hydroxyecdysone (20E), one of the major active  
112 forms of ecdysteroids (Dinan et al., 2001; Kontogiannatos et al., 2015).

113 To gain further information on the effects of BPA on crop pest insects, we used Egyptian  
114 cotton leafworm, *Spodoptera littoralis*, a highly polyphagous species considered one of the most  
115 important pests in Mediterranean countries (Zhang, 1994). We have already demonstrated that *S.*  
116 *littoralis* is sensitive to endocrine disruptors since DEHP can alter post-embryonic development in  
117 this insect (Aviles et al., 2019). The development in insects depends on changes in haemolymph

118 levels of both ecdysteroid hormones, including one major form, 20-hydroxyecdysone (20E), and  
119 sesquiterpenoid juvenile hormones (JHs) (Siaussat et al., 2008). These hormones controlled the  
120 expression of nuclear receptor genes that activate signalling pathways and various physiological  
121 and developmental processes. We already demonstrated that *S. littoralis* can be sensitive to  
122 endocrine disruptors, since DEHP can alter in this insect the post-embryonic development and  
123 metamorphosis, by interfering with ecdysteroid pathways. Therefore *S. littoralis* could be considered  
124 as a fair model to study effects of this EDC on post embryonic development and to analyse the  
125 endocrine mechanisms involved (Aviles et al., 2019). Here, we monitored the effects of BPA  
126 ingestion at low environmental concentrations on several developmental parameters of *S. littoralis*,  
127 such as larval stage duration, larval weight and sex ratio. Effects during the last larval instar were  
128 investigated in more detail, as it is a crucial period preceding the pupal stage and metamorphosis.  
129 Haemolymph titres of 20-hydroxyecdysone, as well as the expression level of several nuclear  
130 receptors involved in the ecdysteroid signalling pathway (EcR, USP, two early-late genes  
131 (Ecdysone-induced protein 75: E75AB and E75D), and the Broad-Complex gene (Br-c)) were  
132 monitored. Our study provides new evidence and precision regarding the events that occur after  
133 BPA exposure in insect crop pests contaminated by food ingestion.

134

## 135 **2. Materials and Methods**

### 136 ***2.1 Insect rearing, BPA treatment and study of post-embryonic development***

137 *S. littoralis* larvae were reared on a semi-artificial diet (Hinks and Byers, 1976) at 23 °C and  
138 60-80% relative humidity and under a 16:8 light:dark cycle. Males and females were identified at the  
139 pupal instar and reared separately. BPA (CAS No. 80-05-7) was purchased from Sigma-Aldrich  
140 (Saint-Quentin Fallavier, France). Twenty-six milligrams of BPA were dissolved first in 250 µL of  
141 ethanol by vortexing and then diluted in water to obtain the 0.5 mM BPA stock solution. The stock  
142 solution was diluted in water and mixed with food to obtain the different final concentrations of BPA,  
143 e.g., 0.01, 0.1, 1 and 25 µg/g of diet. After mixing, the contaminated food was poured into plastic  
144 boxes devoid of BPA, allowed to dry and then kept at 4 °C. A new artificial diet was made each week.  
145 BPA food was first given to third stage larvae, which were then kept individually in small plastic  
146 dishes under the same conditions as described above. A control batch was added to the four treated  
147 batches. Every day, at the end of the photophase, the food was renewed, and the moulting stage  
148 was assessed. Larval mass (Figure S1A), food consumption (Figure S1B) and mortality rate were  
149 monitored daily. As recommended by Farrar et al. (1989), a growth index (GI) for the last larval instar  
150 was calculated using the following formula:  $GI = (W6 - W1) / (6 \times W1)$  with W6 and W1 being the weight  
151 in mg of larvae at the 6<sup>th</sup> and 1<sup>st</sup> day of the last larval instar. The apparition of a cephalic capsule  
152 was assessed every day to determine the instar duration and the beginning of ecdysis. Male and  
153 female pupae were kept separately to determine the pupal stage duration, the sex-ratio and the  
154 percentage of mortality. Haemolymph was collected at each day of the seventh (last) stage (L7) to  
155 measure ecdysteroid levels (see next paragraph). After haemolymph collection, the larvae were

156 pooled by 3 to 5 and frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  in an Eppendorf vial until dissection  
157 of brains and RNA isolation of brains and rest of bodies.

158

## 159 **2.2. Ecdysteroid extraction and measurement**

160 Haemolymph of seventh-instar larvae (L7) was collected in an Eppendorf vial by cutting a  
161 proleg and centrifuged at 9,000 g for 5 min. The supernatant volume was carefully measured, and  
162 transfer to a new tube to add pure methanol (Merck, France, 5 times volume) before vortexing.  
163 Samples were stored at  $-18^{\circ}\text{C}$  until extraction. Then, vials were centrifuged at 9,000 g for 10 min,  
164 the supernatant was collected, and the pellet was reextracted with methanol (250  $\mu\text{L}$ ). The pooled  
165 supernatants were dried under vacuum in a SpeedVac Concentrator (Eppendorf, Hamburg,  
166 Germany). Ecdysteroids were quantified with an enzyme immunoassay (EIA) adapted from the  
167 method of Porcheron et al. (1989), as previously described (Bigot et al., 2012). The polyclonal anti-  
168 ecdysone antiserum L2 (generous gift from Dr. M. De Reggi, Marseille) was used. L2 displayed the  
169 highest affinity towards ecdysone (E) and recognized 20E fivefold less than E in our experimental  
170 conditions (antiserum L2 and enzymatic tracer used respectively at 1/62,500 and 1/100,000 initial  
171 dilution). Dried samples were resuspended in EIA buffer solution. In routine experiments, calibration  
172 curves were generated with 20E (2,000–15.8 fmol/tube), and the results are given as 20E  
173 equivalents fmol/ $\mu\text{L}$  of haemolymph.

174

## 175 **2.3. RNA isolation and cDNA synthesis**

176 Larvae were sampled during the last instar and stored at  $-80^{\circ}\text{C}$  until total RNA extraction by  
177 the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) coupled to RNAeasy Mini kit (Qiagen, USA).  
178 RNA quality was assessed by spectrophotometry at 260 nm and 280 nm (BioPhotometer,  
179 Eppendorf, Hamburg, Germany). Treatment with DNase I (Roche, USA) was performed in  
180 accordance with the manufacturer's instructions. Single-stranded cDNAs were synthesized from 5  
181  $\mu\text{g}$  of RNA with Superscript II Reverse Transcriptase TM (Invitrogen) using the manufacturer's  
182 protocol. For each experimental condition, four to five biological replicates were performed.

183

## 184 **2.4. qRT-PCR**

185 Quantitative real-time PCR analyses were conducted as described in Bigot et al. (2012). The  
186 cycle threshold values (Ct-values) were determined for the reference and candidate genes using  
187 LightCycler@480 software (Roche, France). The average Ct value of each triplicate reaction was  
188 used to normalize the candidate gene expression level to the geometric mean of the reference  
189 gene's level in Q-Gene (Simon, 2003). Seven different genes (Actin, RPL13, Rpl8, GAPDH, tubulin,  
190 ATPase, Ubiquitin) were tested as putative reference genes in all experimental conditions. Specific  
191 qPCR primers for housekeeping genes or targeted genes were designed using Eprimer3 software  
192 (<http://emboss.bioinformatics.nl/cgi-bin/emboss/eprimer3>), and primer annealing temperatures were  
193 optimized using qPCR tests. After BestKeeper analysis (Pfaffl et al., 2004), RPL13 was selected as  
194 the reference gene due to its consistent expression under all experimental conditions (see Table S1



195 for additional information). Mean normalized expression (MNE) was determined with Q-Gene  
196 software (Simon, 2003). We studied the expression variation of nuclear receptors involved in the  
197 ecdysteroid signalling pathway: EcRB, USP, E75AB, E75D and Br-C (see Table S1 for additional  
198 information). Since EcR has two isoforms, A and B, with a common region, we designed the primers  
199 in a specific region of isoform B. A heat map representation of expression levels was then made  
200 using PAST3 software (Hammer et al., 2001) after a log transformation of normalized expression,  
201 centred on the control value.

202

## 203 **2.5. Statistical analyses**

204 Statistical analyses were performed using STATISTICA (StatSoft Enterprise, USA), JMP  
205 (SAS institute, France) and XLstat software (Addinsoft, France). The Shapiro-Wilk test was used to  
206 assess the normality of the data, and Bartlett's test was used to assess the homogeneity of the  
207 variance. The sex ratio between males and females was analysed by comparison of two proportions  
208 by a bilateral test (Monte Carlo method). Duration of stage, size of pupae and ecdysteroid  
209 concentrations in the haemolymph were analysed with Kruskal-Wallis tests; if differences were  
210 found, pairwise comparisons were conducted using a Wilcoxon test. The weights of larvae and qPCR  
211 results were analysed by ANOVA and Tukey's post hoc test. Mortality during larval and pupal stages  
212 was analysed with a Chi-square test.

213

## 214 **3. Results**

### 215 **3.1. BPA affects *S. littoralis* post-embryonic development**

216 Third-instar larvae (L3) were fed every day until pupation with food containing four  
217 concentrations of BPA (0.01, 0.1, 1 and 25 µg of BPA per gram of food). We then recorded mortality  
218 for each stage, the duration of the larval and pupal stages, weight of larvae, size of pupae and the  
219 apparition of abnormality after metamorphosis.

220 Upon the different conditions, larval survival was reduced only for the L7 stage (0.01 BPA  
221 condition), whereas the pupae were affected by three doses (0.01, 0.1 and 25 µg/g) (Figure 1, Chi-  
222 square,  $P < 0.05$ ). No effect was observed on adult stages. No abnormalities of metamorphosis were  
223 observed for all conditions. The different treatments increased the duration of the L5 instar without  
224 any exception (Figure 2, Wilcoxon test,  $P < 0.05$ ), with an average from 3.03 to 3.67 days, compared  
225 to controls with 2.73 days (Figure 2). The two intermediate concentrations (e.g., 0.1 and 1 µg/g of  
226 BPA) had the same effect on the L6 instar (e.g., 3.6 and 3.7 average days, respectively, compared  
227 to the control with 3.4 days) (Figure 2, Wilcoxon test,  $P < 0.05$ ). The observed profile during these  
228 two instars suggests an inverted U-shaped curve with a higher effect at intermediate concentrations  
229 of BPA. Interestingly, a dose-dependent response to BPA on L7 duration was observed with a  
230 progressive decrease of the duration, which was significant for the two highest BPA concentrations  
231 (6.6 and 6.55 average days for 1 and 25 µg/g of BPA compared to the control with 7.04 average  
232 days) (Figure 2, Wilcoxon test,  $P < 0.05$ ). The entire duration of the three larval stages (L5 + L6 +  
233 L7, Figure 2) showed that development times from L5 to L7 were similar among larvae exposed to

234 0.01, 1 and 25 µg/g BPA and controls (e.g., approximately 13.2 days). However, a significant one-  
235 day increase was observed for the larvae exposed to 0.1 µg/g BPA, with an average of almost 24 h  
236 overtime (e.g., 14.13 days, [Wilcoxon test, P < 0.05](#)). A significantly higher growth index was  
237 observed for the L7 larvae fed 1 µg/g of BPA ([Figure 3, Student's t test, P < 0.05](#)). Moreover, these  
238 larvae exhibited a higher weight (2.48 mg) than the control larvae ([Wilcoxon test, P < 0.05, see](#)  
239 [Figure S1A for additional information](#)).

240 The duration of the pupal stage was also disrupted by some BPA concentrations ([Figure 4A](#)).  
241 Thus, a decrease of the duration was observed for males and females obtained from larvae fed 1  
242 µg/g of BPA (16 and 13.8 days, respectively) compared to the corresponding controls (16.5 and 15.25  
243 days) ([Figure 4A, Wilcoxon test, P < 0.05](#)). In contrast, an increased duration was observed for the  
244 female pupae obtained from larvae treated with the higher concentration of BPA (17.25 days in  
245 comparison to the control with 15.25 days) ([Figure 4A, Wilcoxon test, P < 0.05](#)). Larval exposure to  
246 BPA also seems to influence the size of pupae depending on the dose because male pupae obtained  
247 from larvae exposed to 0.1 µg/g of BPA were smaller (average of 12.04 mm) than their controls  
248 (13.01 mm) ([Figure 4A, Wilcoxon test, P < 0.05](#)). Female pupae obtained from larvae exposed to 1  
249 µg/g of BPA were, on the contrary, larger (14.243 mm) than their controls (13.35 mm) ([Figure 4B,](#)  
250 [Wilcoxon test, P < 0.05](#)). Finally, no modification of the sex ratio in pupae was noted regardless of  
251 BPA concentration ([Figure 5](#)).

252

### 253 **3.2. BPA modulates haemolymph concentrations of ecdysteroids in larvae**

254 Treated and control larvae were sampled each day during the last larval instar (L7) to finely  
255 follow the evolution of haemolymph ecdysteroid concentrations. In Lepidoptera, a progressive  
256 increase in concentrations can be observed approximately at the middle of the last larval instar,  
257 leading to a peak of ecdysteroids usually observed before the onset of pupal stage and  
258 metamorphosis. In *S. littoralis*, this peak can indeed be observed at day 6 for control L7 ([Figure 6](#)).  
259 That day, the 20E titre was clearly lower for treated larvae, regardless of the dose of BPA treatment  
260 (e.g., 1763.9, 341.5, 776.2 and 389.2 fmol 20E equiv./µL for the 0.01, 0.1, 1 and 25 µg/g conditions,  
261 respectively, compared to 3277.9 fmol 20E equiv./µL for the controls) ([Figure 6, Wilcoxon test, P <](#)  
262 [0.05](#)). Larvae treated with 0.01 BPA still reached its peak at day 6, as a decrease was observed just  
263 after as for the controls, whereas the increasing phase of the peak of ecdysteroids seemed delayed  
264 to day 7, as we observed an increase in the 20E titre for the other treated larvae.

265

### 266 **3.3. Expression levels of nuclear receptors in larvae**

267 In Lepidoptera, the mid-last larval instar (L7) is usually characterized by a rapid increase of  
268 ecdysteroid production by the prothoracic glands (PGs) ([Satake et al., 1998](#)), as observed here in  
269 *S. littoralis* ([Figure 6](#)). We thus focused on this pivotal period to compare the expression level of  
270 genes involved in the ecdysteroid pathway in “young” L7 larvae (pool of larvae sampled at day 1, 2,  
271 3 or 4; before the onset of ecdysteroids) and “old” L7 larvae (sampled at day 5, which corresponds  
272 to the begin of the increase in ecdysteroid and at days 6 and 7 during the onset of ecdysteroid



273 production). We followed using qPCR the transcript levels of the Ecdysone receptor (EcR), its  
274 heterodimeric partner (USP), two early-late genes (E75AB and E75D), and the Broad-Complex gene  
275 (Br-C) (Figure 7). The expression levels were studied within the brain and the rest of the body since  
276 different hormonal regulations are known to occur in these insect tissues (Truman 2005, Nijhout  
277 2003).

278 For younger larvae, we observed a global decrease in nuclear receptor expression in the  
279 body (without brain) of BPA-treated animals compared to the controls (Fig. 7A, Wilcoxon test,  
280  $P<0.05$ ), with the exception of Br-C transcripts in larvae exposed to 0.01  $\mu\text{g}$  of BPA per gram of food.  
281 The range of variation was approximately 3- to 12-fold less for EcR, 2.5- to 7-fold for USP, 2- to 4-  
282 fold for E75AB, 2- to 8-fold for E75D and approximately 2-fold for Br-C (Fig. 7A and Fig S2, Wilcoxon  
283 test,  $P<0.05$ ). A similar pattern was observed in the brains of these larvae, with a global decrease in  
284 gene expression. Thus, E75D expression was reduced for all BPA conditions, and all genes showed  
285 decreased expression for the 0.01 BPA condition (Fig. 7A and Fig S2, Wilcoxon test,  $P<0.05$ ).  
286 Moreover, we reported a decrease in EcR and USP expression in larvae exposed to 1  $\mu\text{g/g}$  BPA and  
287 of USP and E75AB expression in 25  $\mu\text{g/g}$  BPA-treated larvae. Moreover, we noted that the decrease  
288 amplitude was lower in the brains of younger larvae compared to the body, with only a 1.5- to 3.2-  
289 fold reduction.

290 We observed different expression profiles in the bodies and brains of older larvae. First, we  
291 observed an increase in the level of expression in comparison to younger larvae (data not shown,  
292 Wilcoxon test,  $P<0.05$ ). This increase was correlated with the increase in ecdysteroid levels as  
293 predicted by the Ashburner model (1974). We also observed an increase in gene expression with  
294 discrepancies depending on the genes and BPA treatments. (Figure 7A, Wilcoxon test,  $P<0.05$ ). A  
295 significant increase of EcR and USP was observed for all BPA conditions in the body and brains,  
296 with the exception of the body of larvae exposed to 0.1  $\mu\text{g/g}$  BPA, where no significant difference  
297 with the control was detected. A significant increase in Br-C expression was also observed in the  
298 body of older larvae exposed to 0.1, 1 and 25  $\mu\text{g/g}$  BPA (Figure 7A, Wilcoxon test,  $P<0.05$ ).  
299 Interestingly, for these conditions of BPA, Br-C expression was not affected in the brain of the  
300 corresponding larvae, whereas we observed a decrease in its expression in the brains of the larvae  
301 exposed to 0.01  $\mu\text{g/g}$  BPA. This difference in the profiles between brains and bodies was also  
302 observed with the expression of E75AB. More specifically, an increase in expression was observed  
303 in older larvae brains exposed to 1 and 25  $\mu\text{g/g}$  BPA, while a decrease was observed in the body of  
304 the 25  $\mu\text{g/g}$  BPA older larvae. The level of expression of all genes was between 3 and 6-fold higher  
305 than the control level (Figure 7A, Wilcoxon test,  $P<0.05$ ).

306

#### 307 4. Discussion

308 Studies on BPA effects in terrestrial insects are thus far only available for a few species, e.g.,  
309 *M. domestica* (Izumi et al., 2008), *D. melanogaster* (Atli, 2013, Weiner et al., 2014, Williams et al.,  
310 2014, Kaur et al. 2015, Quesada-Calderón et al. 2017) and *S. nonagrioides* (Michail et al., 2012;  
311 Kontogiannatos et al., 2015). These reports noted various effects, such as larval weight variations,

312 modification of sex ratio, faster pupariation or abnormal phenotypes. Other studies highlighted the  
313 impact on gene expression of nuclear receptors. However, such parameters were never observed  
314 in the same study. Here, our integrative approach allowed us to compare the effects of various doses  
315 of BPA on key parameters of post-embryonic development.

316 After BPA exposure of our larvae of *S. littoralis* by ingestion from the 3rd larval instar (L3),  
317 we first observed a significant mortality increase of the resulting L7 larvae and pupae but not in adults  
318 (Figure 1). L7 larvae were only affected by the lowest BPA dose (e.g., 0.01 µg/g), whereas almost  
319 all doses used induced an important mortality during the pupal stage. This highlights the fact that  
320 BPA effects on insect survival could be highly variable depending on the contaminant dose and that  
321 this effect could be delayed in time by inducing an effect on a stage, although the exposure was  
322 during the previous stage. Few studies have reported the effect of BPA on insect mortality, and in  
323 most cases, mortality monitoring was performed on the same insect developmental stage as the  
324 treated one. Low or no mortality was observed in *Chironomus riparius* larvae (Morales et al., 2011).  
325 Similarly, BPA had few impacts on *M. domestica* survival (Izumi et al., 2008). In *S. nonagrioides*  
326 larvae, treatments with low BPA concentrations (from 1 to 100 µg/L; Michail et al., 2012) did not  
327 induce significant mortality, whereas continuous exposure to higher doses ((e.g., 1 and 10 mg/L of  
328 BPA) had an effect. Eventhough Michail et al. (2012) used a different protocol for artificial diet  
329 preparation than us, we can estimate that the mortality observed in this latter case was for much  
330 higher BPA concentrations than in the present study. The significant increase of mortality observed  
331 here in *S. littoralis* L7 larvae for the lowest dose of BPA, but not for the higher ones, is in line with  
332 the known nonlinear effects of EDC in animals (reviewed in Vandenberg et al. 2009).

333 Several disruptions during *S. littoralis* post-embryonic development were induced by BPA  
334 treatments. An increase in L5 and L6 instar durations was observed (Figure 2) following almost all  
335 BPA treatments. This finding confirms results obtained previously in *S. nonagrioides* by  
336 Kontogiannatos et al. (2015) since they reported an extended transition period between larval and  
337 pupal instars after contamination by various doses (e.g., 1, 10 and 100 µg/L) of BPA. However, we  
338 also observed this increase in *S. littoralis* larvae treated with lower concentrations (e.g., 0.01 or 0.1  
339 µg/g) of BPA, i.e., at doses close to those measured in plants and that *S. littoralis* could frequently  
340 encounter. This effect of the lower concentration found in plants was unexplored to date in a crop  
341 pest. In contrast, in the last larval instar (e.g., L7), the stage duration was shortened for 1 and 25  
342 µg/g of BPA. This result appeared consistent with the study of Weiner et al. (2014), in which BPA  
343 induced an earlier onset of pupariation during post-embryonic development of *D. melanogaster*.  
344 Interestingly, only the concentration of 0.1 µg/g BPA had an effect on the whole larval stage duration  
345 (when compiling durations from L5 up to L7 end), suggesting that the observed extended durations  
346 of L5-L6 instars were balanced by the reduction of the L7 instar. A precise measure of each instar  
347 duration is thus required to detect such a disruption. Interestingly, as observed by Kontogiannatos  
348 et al. (2015), BPA treatments can also affect pupal stage duration in *S. nonagrioides*. However, we  
349 observed differences between males and females for the two higher concentrations of BPA, showing  
350 that this disruption can be sex specific (Figure 4A).

351 Insect growth is under endocrine control and is one of the most studied endpoints as an  
352 endocrine disruption effect (Soin & Smaghe, 2007). BPA also disrupted the growth of *S. littoralis*  
353 larvae and pupae; at 1 µg/g BPA, we observed a higher growth index in L7 larvae (Figure 3). Our  
354 results confirmed that low environmental doses of BPA can stimulate the growth of larvae, as  
355 demonstrated in larvae of *D. melanogaster* exposed to very low doses of BPA (e.g., 0.1 mg/L).  
356 However, BPA appeared to induce the opposite effect on the growth of pupae (Figure 4B) since we  
357 observed a longer body length of pupae in females treated with 1 µg/g BPA and a shorter length in  
358 0.1 µg/g BPA males.

359 One very interesting point is that growth stimulation in larvae and female pupae was observed  
360 under BPA conditions that did not induce mortality. This opposite dose-dependent effect was also  
361 reported in other species. Thus, in *M. domestica*, exposure to 100 ng/g BPA induced an increase in  
362 pupal weight and length, whereas at 1000 ng/g, the opposite effect was observed (Izumi et al., 2008).  
363 Even if the mechanism of growth disruption by BPA in terrestrial insects is not completely  
364 understood, some results obtained in *D. melanogaster* provide explanations (Williams et al., 2014).  
365 Indeed, BPA appears to affect lipid metabolism during larval starvation by inhibiting insulin-like  
366 peptide expression and possibly by inhibiting larval lipolysis capacities during starvation. All these  
367 disruptions induced a significant increase in lipid content that affects larval weight and the energetic  
368 stocks that insects will use during metamorphosis. We recently highlighted an increase in energetic  
369 metabolites during the last larval instar in *S. littoralis* to prepare for the pupal period and  
370 metamorphosis (Aviles et al., 2019). This production of metabolites can be disrupted by phthalates  
371 and, likely, by other endocrine disruptors, such as BPA (Aviles et al., 2019).

372 We did not observe any body formation defects during *S. littoralis* development, whereas  
373 Kontogiannatos et al. (2015) observed an increased percentage of abnormalities with increasing  
374 BPA concentrations. As the sex of the larvae is impossible to determine by an external observation,  
375 we studied the effect on the sex ratio at the pupal stage since external specific patterns in the  
376 posterior part can be observed in the lepidopteran. Thus, no difference in the sex ratio of *S. littoralis*  
377 was observed following BPA exposure (Figure 5), whereas BPA appeared to increase the number  
378 of males in comparison to females in terrestrial insects such as *M. domestica* (Izumi et al., 2008) or  
379 in aquatic species such as *C. riparius* (Lee & Choi, 2007). DEHP, another endocrine disruptor,  
380 appeared to induce different effects in *S. littoralis*. Only the two highest non-environmental doses of  
381 DEHP induced mortality (Aviles et al., 2019). The body weight was not affected even if some  
382 disruption of energetic metabolites was observed in larvae exposed to DEHP.

383 In *S. littoralis*, the typical ecdysteroid peak preceding the pupal moult occurred during the 6th  
384 day of the last larval instar (Aviles et al., 2019). However, in comparison, we observed here that the  
385 increasing concentration of BPA seems to progressively induce a decrease in the ecdysteroid peak  
386 intensity and then a 24 h delay for the three highest concentrations tested (Figure 6). We also  
387 observed some differences in expression for the nuclear receptors of the ecdysteroid signalling  
388 pathway, depending on the period of last larval instar: we observed, during the second phase of this  
389 larval stage, that BPA globally increases the expression of EcR and USP, the 20-hydroxyecdysone

390 receptor dimer, in the bodies and brains of almost all treated larvae (Figure 7). This increase in  
391 expression is quite classic, as reported in *S. nonagrioides* or *C. riparius* (Kontogiannatos et al.,  
392 2015; Planello et al. 2008).

393 However, our results showed a difference between the younger and older larvae. During the  
394 first days of the lepidopteran last larval instar, PGs found in the larvae are inactive and insensitive  
395 to the prothoracic hormone (PTTH) because of the presence of the juvenile hormone (JH) (Gu et al.,  
396 1997). The absence of JH from the 4<sup>th</sup> day allows PTTH to induce the production of ecdysteroids by  
397 the PGs and the appearance of a haemolymph peak at the 6<sup>th</sup> day in *S. littoralis* (Satake et al., 1998).  
398 The increased ecdysteroid levels induce a genetic reprogramming of larvae and activation of a pupal  
399 commitment leading to metamorphosis (Nijhout, 1994). Our results showed that the same treatment  
400 with BPA can induce very different modifications of nuclear receptor expression depending on the  
401 period of the last instar. The second period shows an increasing concentration of ecdysteroid and  
402 induction of the ecdysteroid signalling pathway in hormone-targeted cells (Siaussat et al. 2008). In  
403 this context, BPA exposure increases the expression of the first molecular actors of the signalling  
404 cascade. This result is very different compared to the first period of the last larval instar, where a  
405 general decrease in expression was observed in BPA-exposed larvae. During this period, insect  
406 cells are not genetically ready to induce the increase of ecdysteroids, leading to a different effect of  
407 BPA on gene expression.

408 The comparison between brains and bodies highlighted the different genetic programmes  
409 activated in insect tissues. Indeed, BPA exposure also induces a modification of the expression of  
410 ecdysteroid pathway early genes such as E75AB, E75D and Br-C (Huet et al., 1993). However, our  
411 results showed that the involved early genes are differentially expressed in brains compared to the  
412 rest of the bodies. Br-C expression is significantly increased during the second period in the bodies  
413 of larvae for almost all BPA conditions, whereas no modification was observed in larvae brains. In  
414 brains, we observed an increase in the expression of E75AB for the two highest concentrations of  
415 BPA, whereas it was not affected in the rest of the bodies.

416 Whereas BPA was clearly demonstrated to have a strong affinity for PPAR and RXR, the  
417 homologs of EcR and USP in vertebrates (Sharma et al., 2018), there is currently no obvious  
418 evidence of BPA binding on these two nuclear receptors. The increase in EcR and USP expression,  
419 such as in *C. riparius* or *S. nonagrioides*, is the first evidence (Kontogiannatos et al., 2015; Planello  
420 et al. 2008). Indeed, during the second period of the last instar, feedback regulation of early and  
421 early-late genes on the EcR and USP genes can occur, leading to an increase in their expression  
422 levels (Ashburner, 1974; Huet et al., 1995). Moreover, Tarrant et al. (2011) demonstrated that, at  
423 high doses, BPA can fully outcompete ponasterone A that normally binds to the EcR of *Homarus*  
424 *americanus*. Kontogiannatos et al. (2015) used a genetically modified insect cell line allowing the  
425 detection of all compounds able to interact with the EcR/USP complex through GFP activation  
426 (Swevers et al. 2004). A previous study demonstrated that BPA acts as a weak agonist of the  
427 ecdysteroids (Dinan et al. 2001). Even if these two studies did not allow us to demonstrate the direct  
428 binding of BPA on EcR/USP, they provided strong evidence for it. In our study, we also did not

429 provide a functional demonstration of this binding, but one can hypothesize that BPA in *S. littoralis*  
430 could interact with EcR/USP, leading to modification of the gene expression of actors of the genetic  
431 cascade that have to be expressed following the genetic programme of the corresponding tissue.

432 Our results also highlighted that it could be difficult to correlate the expression levels of  
433 nuclear receptors and ecdysteroid titration. This may be due to the ability of BPA to induce different  
434 mechanisms of disruption. On one hand, BPA as an endocrine disruptor could probably act on  
435 (ecdys)steroidogenesis by disrupting the activity of enzymes involved in the synthesis of endogenous  
436 steroid hormones and their metabolism. On the other hand, the binding of BPA on ecdysteroid  
437 receptors can prevent endogenous ecdysteroids from binding to and activating the nuclear signalling  
438 pathway or leading to important changes in the kinetics and levels of genes involved in the nuclear  
439 receptor signalling pathways (Dinan et al.,2001). The best demonstration of this double effect is the  
440 decrease of the last larval instar duration, whereas the peak of ecdysteroid appeared to be delayed  
441 by 24 h. The hormonal peak usually precedes the pupal moult by 24 or 48 hours. However, this is  
442 not the case here, showing that the binding of BPA on nuclear receptors could mimic the effects of  
443 circulating hormones. Thus, it is crucial to carefully analyse the effects of EDC on insect development  
444 physiology on the two levels of analysis to obtain a complete understanding of the disruption.

445

## 446 **5. Conclusion**

447 Altogether, our results showed that BPA exposure can induce endocrine disruption in *S.*  
448 *littoralis* and the benefits of an integrative approach allowing us to investigate several levels through  
449 the entire life cycle of exposed animals and at a wide spectrum of concentrations. Thus, two different  
450 patterns appeared in our experiments. On one hand, we observed an important mortality caused by  
451 BPA used at environmental doses found in plants potentially attacked by *S. littoralis*. Even if BPA  
452 was ingested by larvae, the effect on mortality rate was mainly observed during the pupal stage,  
453 showing a delay for the negative BPA effects to be revealed. Our results provide some explanations  
454 for this mortality since BPA induces disruptions of ecdysteroid concentrations and nuclear receptor  
455 expression. A disruption of the temporal dynamics and levels of endocrine regulation factors seems  
456 to induce deleterious effects in *S. littoralis*. On the other hand, 1 µg/g of BPA did not increase the  
457 mortality rate in any instar but induced a shorter pupal stage, leading to an earlier emergence of  
458 adults. We did not investigate the effects of BPA on JH levels as performed in *M. domestica* by Izumi  
459 et al. 2008. This experiment could be very interesting to see if this other important family of hormones  
460 could also be disrupted during larval development. The hormonal system crucially controls  
461 metamorphosis and histogenesis of various vital organs of adults. A more focused study on potential  
462 abnormalities that could be induced by BPA would be interesting. Moreover, ecdysteroids and JHs  
463 are involved in the control of reproduction and mating processes in insects. Thus, to understand the  
464 consequences of lethal and non-lethal doses of BPA on adults, we will conduct an exhaustive study  
465 on different physiological parameters in exposed males and females of *S. littoralis*.

466



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648  
649

#### 650 Legends

651 Figure 1. Effect of BPA on the mortality of the larval, pupal, and adult stages. Percentages of  
652 mortality of BPA-contaminated insects were compared to the percentage of control animals. N for  
653 each condition is indicated under the corresponding percentage of mortality. Asterisks indicate  
654 significant differences among means in comparison to controls (Chi-square, \* P<0.05; \*\* P<0.01;  
655 \*\*\* P<0.001).

656

657 Figure 2. Effect of BPA on the duration of the 5<sup>th</sup>, 6<sup>th</sup> and 7<sup>th</sup> larval instars. Instar durations of BPA-  
658 contaminated larvae were compared with the values of control larvae (N= from 30 to 110 for each  
659 condition, depending on the larval stage). L5+L6+L7 correspond to the increase in the duration of  
660 the 5<sup>th</sup>, 6<sup>th</sup> and 7<sup>th</sup> larval instars. Each point represents the mean values ± SEM. Asterisks indicate  
661 significant differences among means in comparison to controls (Wilcoxon test, \* P<0.05; \*\* P<0.01;  
662 \*\*\* P<0.001).

663

664 Figure 3. Effect of BPA on larval growth. The growth index was calculated using the following  
665 formula:  $GI = (W6 - W1) / (6 \times W1)$  with W6 and W1 being the weight in mg of larvae at the 6th and  
666 1st day of the last larval instar. Each point represents the mean values ± SEM (N= 10 biological  
667 replicates with the weight for each replicate of 10 larvae for each condition and day). Asterisks  
668 indicate significant differences among means in comparison to controls (Student's t test, \* P<0.05).

669

670 Figure 4. Effect of BPA on the duration of the pupal instar (A) and the length of the pupae (B). All  
671 the durations or lengths of male or female pupae obtained from BPA-contaminated larvae were  
672 compared to the control pupae of the same sex (n=50-60 for duration for each condition, N=10 for



673 length of pupae). Each point represents the mean values  $\pm$  SEM. Asterisks indicate significant  
674 differences among means in comparison to controls (Wilcoxon test, \*  $P < 0.05$ ; \*\*\*  $P < 0.001$ ).

675

676 Figure 5. Effect of BPA on the sex ratio repartition at pupal stages. The sex was determined after  
677 formation of the pupa based on the external patterns in the posterior part that were different  
678 according to sex. All the percentages of male and female pupae obtained from BPA-contaminated  
679 larvae were compared to the percentage of controls (N=637 to 712). Each point represents the  
680 mean values  $\pm$  SEM.

681

682 Figure 6. Haemolymph ecdysteroid titres during the seventh larval instar in BPA-contaminated or  
683 control larvae (N=8). Ecdysteroid concentrations, expressed in fmol 20E equiv./ $\mu$ L, were quantified  
684 by EIA. Each point represents the mean values  $\pm$  SEM. Points with a different letter are  
685 significantly different (Wilcoxon test). Points in the square are significantly similar.

686

687 Figure 7. Heat map visualization of the expression of nuclear receptors (EcR, USP, E75D, E75AB,  
688 Br-C) in the bodies and brains of younger and older contaminated or control larvae (CT). The  
689 younger larvae correspond to larvae sampled during the 1<sup>st</sup> to 4<sup>th</sup> day of the last larval instar (N=6  
690 for each condition). The older larvae from the 5<sup>th</sup> to 7<sup>th</sup> day of the last larval instar (n=6 for each  
691 condition). Expression levels were quantified by quantitative PCR. A large mean normalized  
692 expression (MNE) value was used to construct the heat map (control was centred to 2). (B)  
693 Schematic representation of the ecdysteroid pathway. After the binding of 20E to EcR and USP  
694 proteins, the complex can enter the nucleus and, by fixation, on the Ecdysone Response Element  
695 (EcRE) induce the expression of others nuclear receptors of the genetic cascade (i.e. E75AB,  
696 E75D, Br-C). Asterisks indicate significant differences among means in comparison to controls  
697 (Wilcoxon test, see Fig S2 and S3 for details on significant differences).

698

699 Figure S1. Effect of BPA on the fresh weight of larvae of the last instar after continuous ingestion  
700 of food contaminated with various concentrations of DEHP. The weight of the larvae was  
701 measured daily from the 1<sup>st</sup> to the 6<sup>th</sup> day. (A) Daily measurement of larval weight. Each point  
702 represents the mean values  $\pm$  SEM (N= 10 biological replicates with the weight for each replicate  
703 of 10 larvae for each condition and day). (B) Graphical representation of food consumption of  
704 larvae in grams. Each point represents the mean of values with the SEM.

705

706 Figure S2. Normalized expression of nuclear receptors (EcR, USP, E75D, E75AB, Br-C) in brains  
707 of contaminated or control young larvae (A) (from the 1<sup>st</sup> to 4<sup>th</sup> day) and older larvae (B) (from the  
708 5<sup>th</sup> to 7<sup>th</sup> day) of the last larval instar (N=6 for each condition). Expression levels were quantified by  
709 quantitative PCR. Each point represents the mean values  $\pm$  SEM. Bars with asterisks indicate  
710 significant differences among means in comparison to controls (Wilcoxon test, \*  $P < 0.05$ ; \*\*  $P < 0.01$ ;  
711 \*\*\*  $P < 0.001$ ).

712

713 Figure S3. Normalized expression of nuclear receptors (EcR, USP, E75D, E75AB, Br-C) in bodies  
714 (without brains) of contaminated or control larvae from the first part (A) (larvae from the 1<sup>st</sup> to 4<sup>th</sup>  
715 day) and second part (B) (larvae from the 5<sup>th</sup> to 7<sup>th</sup> day) of the last larval instar (N=6 for each  
716 condition). Expression levels were quantified by quantitative PCR. Each point represents the mean  
717 values  $\pm$  SEM. Bars with asterisks indicate significant differences among means in comparison to  
718 controls (Wilcoxon test, \* P<0.05; \*\* P<0.01; \*\*\* P<0.001).

719

720 Table S1. Sequences of the primers used for RT-qPCR amplification in *Spodoptera littoralis* larvae.

721 F: forward primer; R: reverse primer

Fig 1

	µg of BPA / g of food				
	Controls	0.01	0.1	1	25
L7	25 (N=332)	35.8 ** (N=312)	27.8 (N=359)	31 (N=387)	31.42 (N=366)
pupae	33.45 (N=284)	52.54 *** (N=236)	45.01** (N=293)	38.17 (N=317)	43.72* (N=295)
Adult males	27.65 (N= 94)	33.76 (N=77)	38.46 (N=65)	36.48 (N=74)	28.40 (N=88)
Adult females	25 (N=88)	25.27 (N=91)	28.98 (N=69)	25.55 (N=90)	25 (N=96)

Fig 2

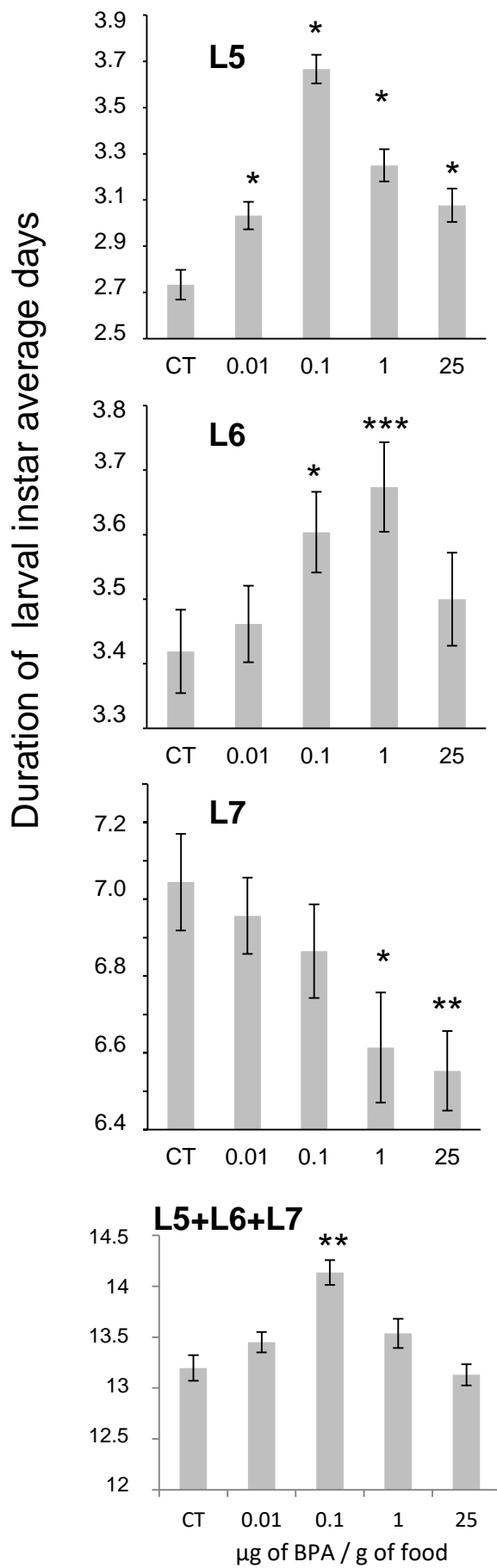


Fig 3

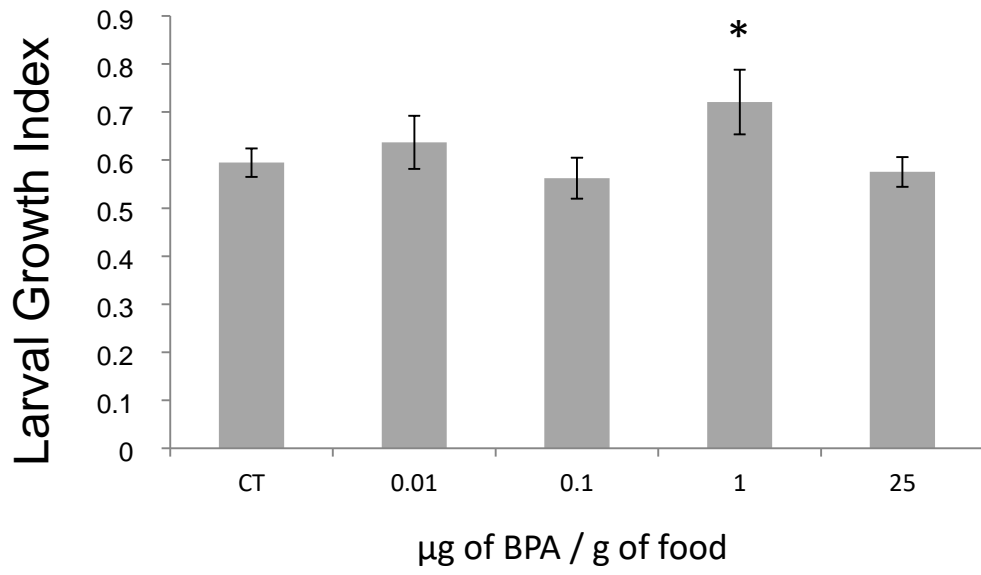




Fig 4

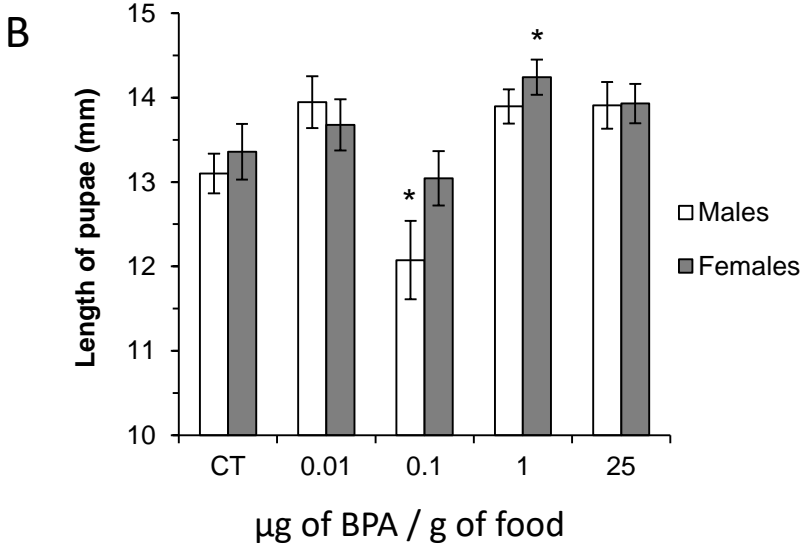
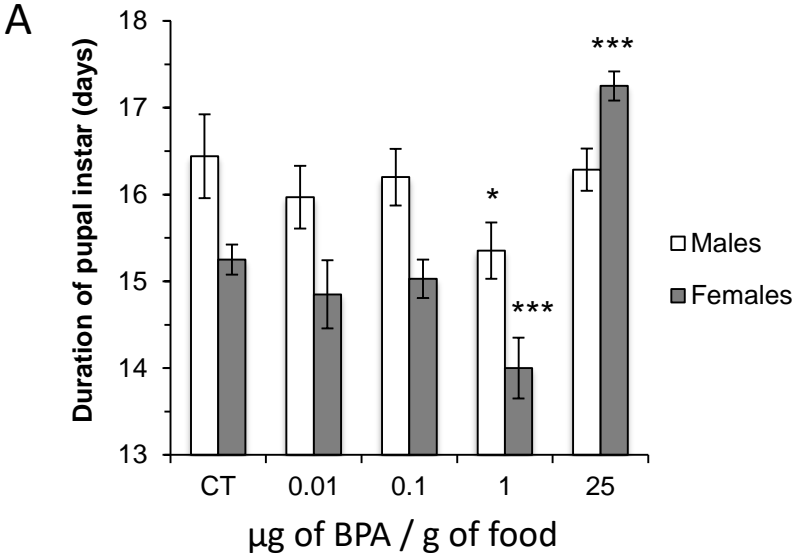


Fig 5

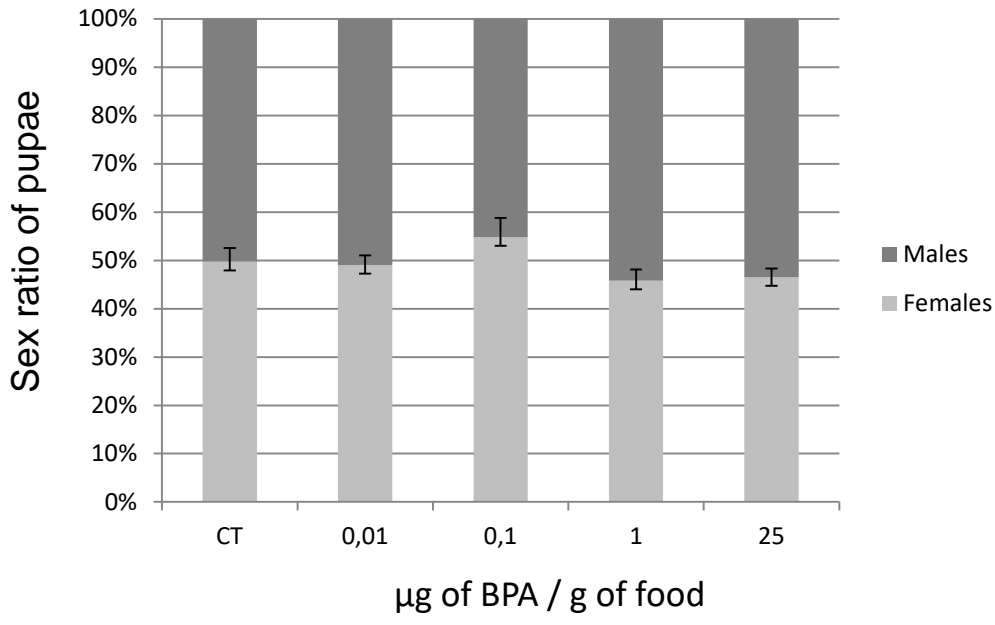
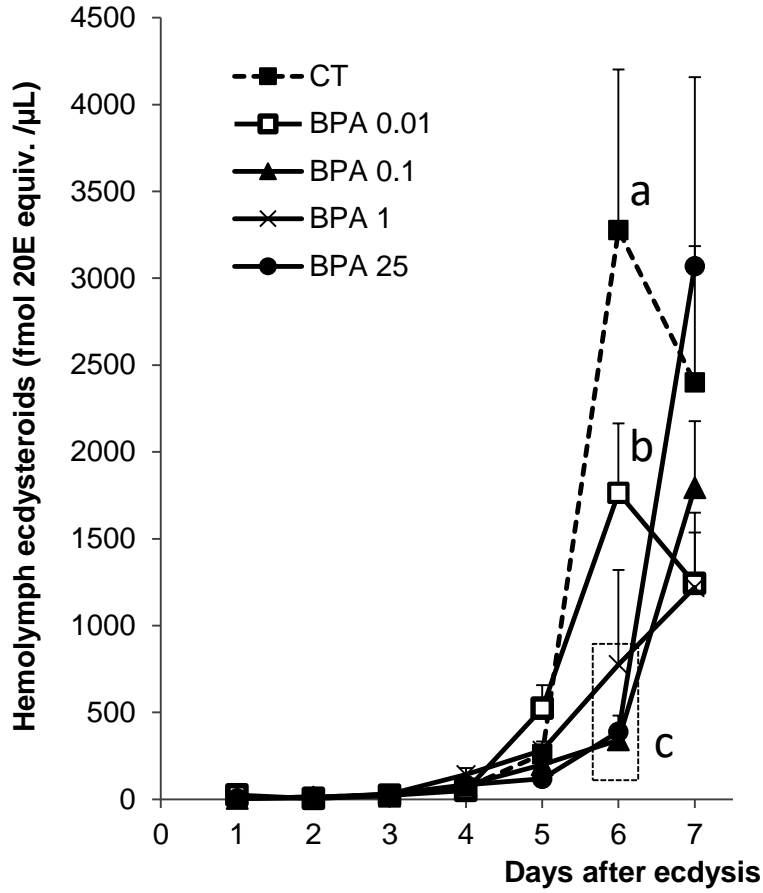


Fig 6



# Fig 7

