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

Annick Maria¹, §, Aude Malbert-Colas^{1,§}, Isabelle Boulogne^{1,2}; Virginie Braman¹, Constance Boitard¹, Matthieu Dacher¹, Thomas Chertemps¹, Martine Maibeche¹, Catherine Blais¹, David Siaussat^{1,*}

Author affiliations:

1 Sorbonne Université, INRA, CNRS, IRD, UPEC, Institut d'Ecologie et des Sciences de l'Environnement de Paris (iEES-Paris), F75005 Paris, France

2 Normandie Université, UNIROUEN, Laboratoire Glyco-MEV EA 4358, Fédération de Recherche "Normandie Végétal" FED 4277, 76000 Rouen, France

§ These two authors are considering as co-first authors

Author contributions:

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

* Corresponding author:

Institut d'Ecologie et Sciences de l'Environnement de Paris – Sorbonne Université, INRA, CNRS, IRD, UPEC

Email address: david.siaussat@sorbonne-universite.fr

Present address: Sorbonne Université, 4 place Jussieu, Campus Pierre et Marie Curie – Tours 44-45, 3ème étage, boîte courrier 237, 75005 Paris (France)

Abstract:

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

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

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

1. Introduction

Endocrine-disrupting chemicals include a variety of chemical compounds that may interfere with the endocrine system of organisms, their descendants, or (sub) populations. Bisphenol A (BPA, CAS No. 80-05-7), a chemical used in various plastic consumer products, is now considered a major pollutant in several countries (Andrady & Neal 2009, Bauer & Herrmann 1997; Staples et al., 2002). BPA is currently included in the candidate list of substances of very high concern for authorization, as in the REACH Regulation. BPA is mostly resistant to environmental degradation (half-life from 1 day to 12 months in water and soil) (Howard, 1989) and can be widely dispersed in the environment through different ways, such as sewerage systems, waste disposal and spraying of contaminated water (Fromme et al., 2002; Porter & Janz, 2003; Wang et al., 2011; Casals-Casas & Desvergne, 2011). Little is known about BPA atmospheric distribution and transport; however, different ranges of concentrations were measured in the atmospheric aerosols from urban, rural, marine and polar regions from 1 to 17400 pg/m³ (Fu & Kawamura, 2010; Salapasidou et al., 2011).

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

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

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

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

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

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

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

2. Materials and Methods

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

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

pooled by 3 to 5 and frozen in liquid nitrogen and stored at –80 °C in an Eppendorf vial until dissection of brains and RNA isolation of brains and rest of bodies.

2.2. Ecdysteroid extraction and measurement

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

2.3. RNA isolation and cDNA synthesis

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

2.4. qRT-PCR

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

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

2.5. Statistical analyses

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

3. Results

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

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

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

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

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

3.2. BPA modulates haemolymph concentrations of ecdysteroids in larvae

Treated and control larvae were sampled each day during the last larval instar (L7) to finely follow the evolution of haemolymph ecdysteroid concentrations. In Lepidoptera, a progressive increase in concentrations can be observed approximatively at the middle of the last larval instar, leading to a peak of ecdysteroids usually observed before the onset of pupal stage and metamorphosis. In *S. littoralis*, this peak can indeed be observed at day 6 for control L7 (Figure 6). That day, the 20E titre was clearly lower for treated larvae, regardless of the dose of BPA treatment (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, respectively, compared to 3277.9 fmol 20E equiv./µL for the controls) (Figure 6, Wilcoxon test, $P < 0.05$). Larvae treated with 0.01 BPA still reached its peak at day 6, as a decrease was observed just after as for the controls, whereas the increasing phase of the peak of ecdysteroids seemed delayed to day 7, as we observed an increase in the 20E titre for the other treated larvae.

3.3. Expression levels of nuclear receptors in larvae

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

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

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

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

4. Discussion

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

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

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

Several disruptions during *S. littoralis* post-embryonic development were induced by BPA treatments. An increase in L5 and L6 instar durations was observed (Figure 2) following almost all BPA treatments. This finding confirms results obtained previously in *S. nonagrioides* by Kontogiannatos et al. (2015) since they reported an extended transition period between larval and pupal instars after contamination by various doses (e.g., 1, 10 and 100 µg/L) of BPA. However, we also observed this increase in *S. littoralis* larvae treated with lower concentrations (e.g., 0.01 or 0.1 µg/g) of BPA, i.e., at doses close to those measured in plants and that *S. littoralis* could frequently encounter. This effect of the lower concentration found in plants was unexplored to date in a crop pest. In contrast, in the last larval instar (e.g., L7), the stage duration was shortened for 1 and 25 µg/g of BPA. This result appeared consistent with the study of Weiner et al. (2014), in which BPA induced an earlier onset of pupariation during post-embryonic development of *D. melanogaster*. Interestingly, only the concentration of 0.1 µg/g BPA had an effect on the whole larval stage duration (when compiling durations from L5 up to L7 end), suggesting that the observed extended durations of L5-L6 instars were balanced by the reduction of the L7 instar. A precise measure of each instar duration is thus required to detect such a disruption. Interestingly, as observed by Kontogiannatos et al. (2015), BPA treatments can also affect pupal stage duration in *S. nonagrioides*. However, we observed differences between males and females for the two higher concentrations of BPA, showing 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 & Smagghe, 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. nonagroides* 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 4th day allows PTTH to induce the production of ecdysteroids by
397 the PGs and the appearance of a haemolymph peak at the 6th 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. nonagroides*, 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

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

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

5. Conclusion

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

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Legends

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

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

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

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

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

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

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

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

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

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

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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 1st to 4th
715 day) and second part (B) (larvae from the 5th to 7th 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

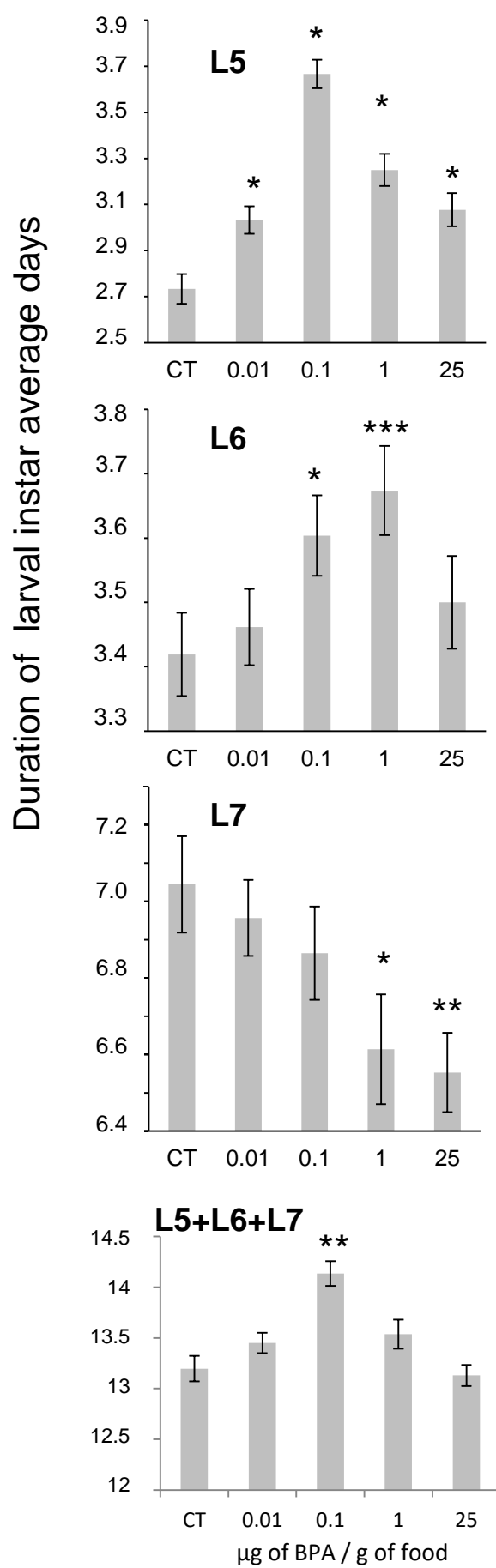


Fig 3

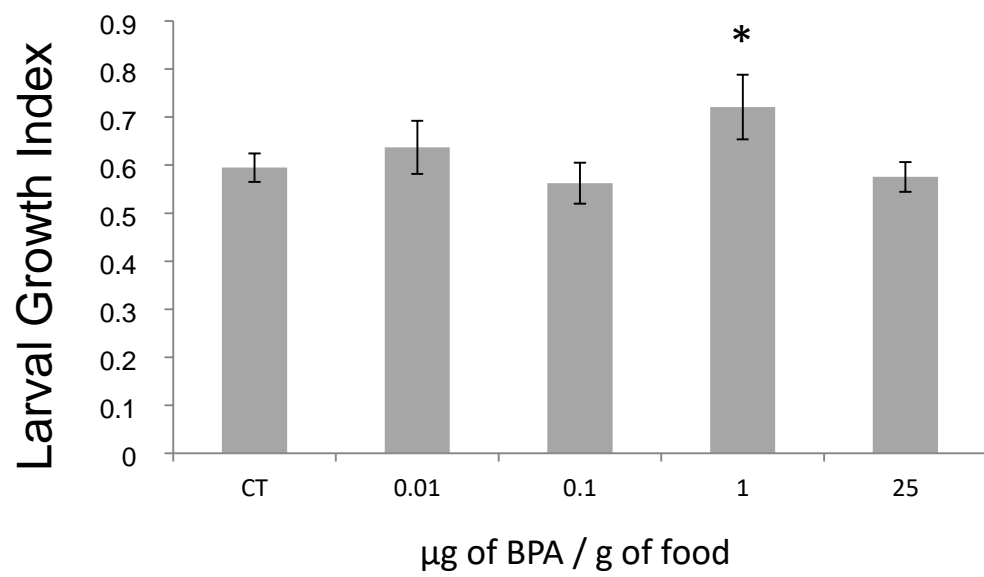


Fig 4

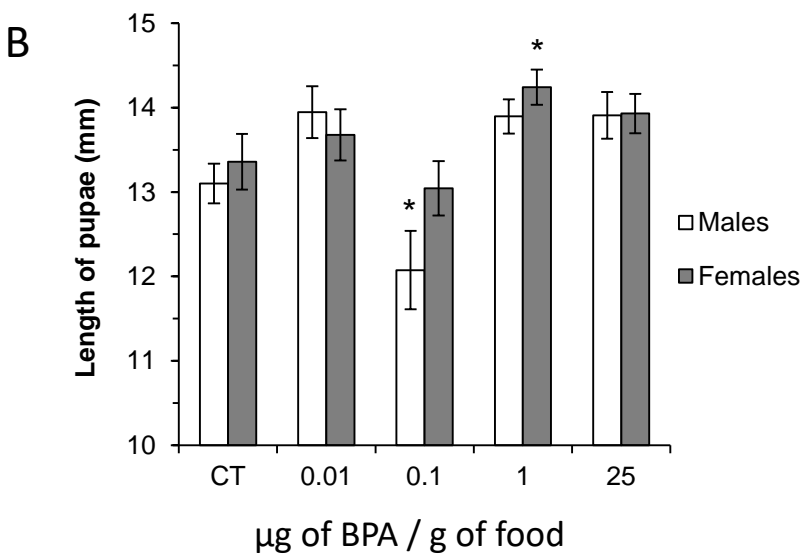
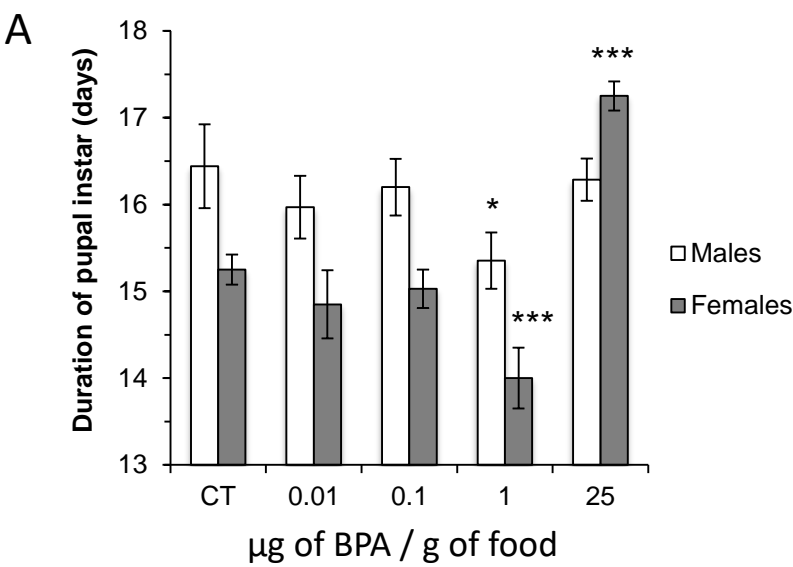


Fig 5

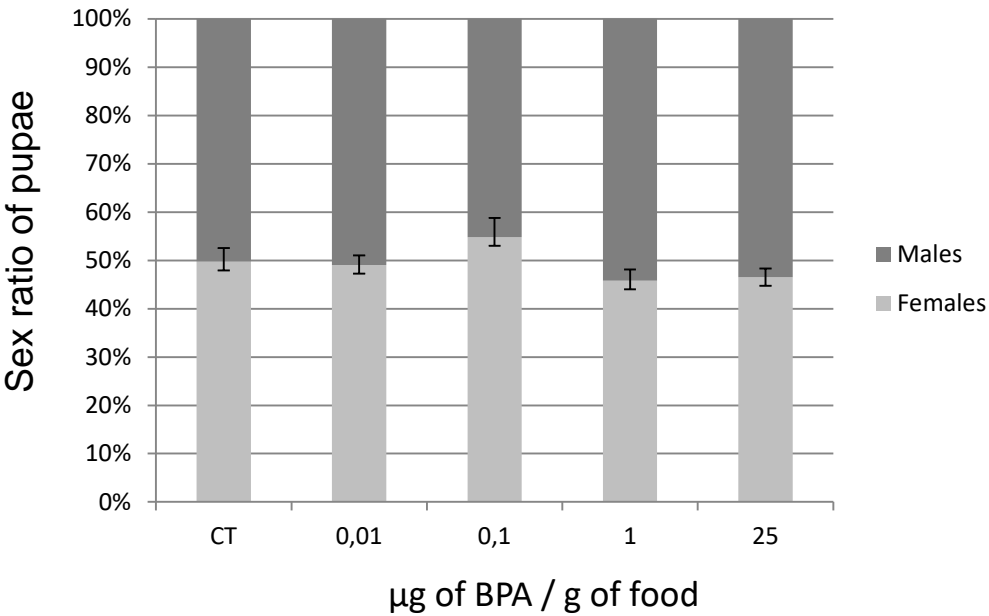


Fig 6

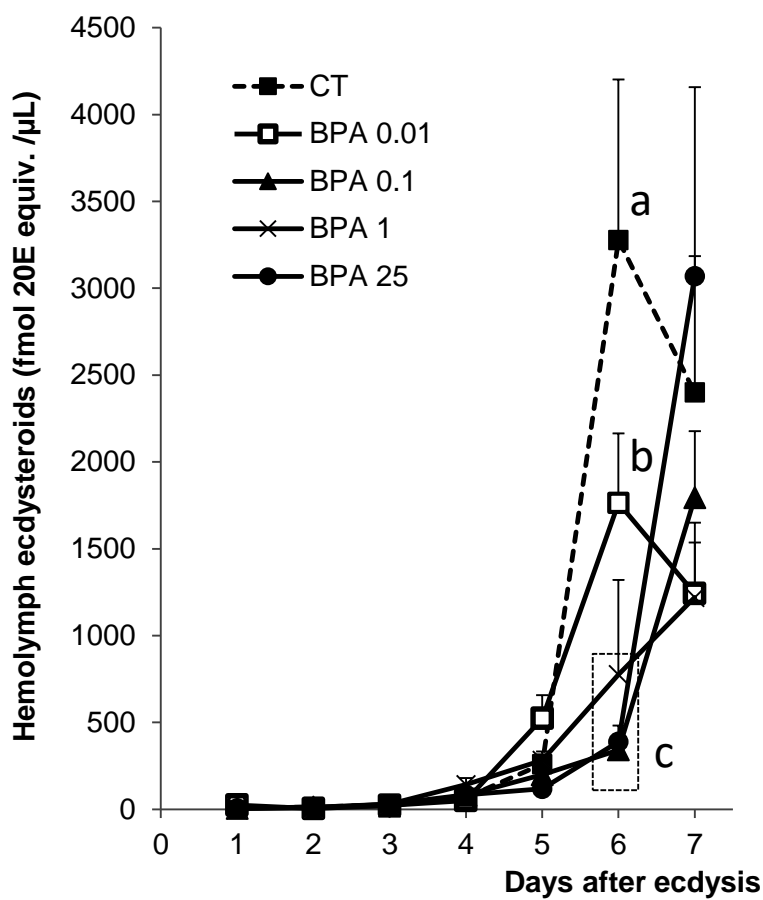


Fig 7

