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Effects of DEHP on post-embryonic development, nuclear receptor expression, metabolite and ecdysteroid concentrations of the moth *Spodoptera littoralis*

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Abstract:

Di (2-ethylhexyl) phthalate (DEHP) is recognized in vertebrates as an Endocrine Disrupting Chemical (EDC). DEHP can alter steroid hormones production, development, reproduction and behavior in vertebrates. Only few studies investigated DEHP effects on insects. However, some recent studies on aquatic insects showed that DEHP could also act as an EDC by interfering with the signaling pathways of ecdysteroids, the main hormones involved in the control of insect post-embryonic development and physiology. The aim of the study was to investigate (1) the fate of DEHP within a terrestrial insect species by exposing larvae to food containing a wide range of DEHP concentrations and (2) the effects of this chemical on their post-embryonic development and metamorphosis, by using a multi-level approach. DEHP was shown to be present both in larvae and resulting stages, with higher concentrations in chrysalises and adults than in larvae. DEHP concentrations also decreased at the end of the last larval instar, suggesting the metabolic transformation or excretion of this chemical during this time. Only the two highest DEHP doses induced higher insect mortality, whereas low and intermediate concentrations increased larval food consumption without affecting body weight. Metabolic profiles showed that in control insects, the last three days before metamorphosis correspond to a metabolic transition, but with time-dependent changes in treated insects. Interestingly, DEHP treatments also alter both hemolymphatic ecdysteroid titers and expression levels of ecdysteroid response genes. These results confirm that DEHP can alter insect post-embryonic development and metamorphosis, by interfering with ecdysteroid pathways.

Keywords: Bis(2-ethylhexyl)phthalate, metabolic profiling, insect, environmental pollution, development

Introduction

53 Endocrine disrupting compounds (EDCs) are chemicals that interfere with the body's
54 endocrine system, and produce adverse developmental, reproductive, neurological and/or immune
55 effects in both humans and wildlife (Matthiessen et al., 2018). EDCs encompass a wide range of
56 chemical substances, including pharmaceuticals, pesticides and plastic compounds. Among plastic
57 compounds, Di(2-ethylhexyl) phthalate (DEHP) is produced in very high quantities and found in
58 many mass-produced products including medical devices, food packaging, perfumes, cosmetics,
59 flooring materials (Bauer & Herrmann 1997; Staples et al., 2002; Andrady & Neal 2009). DEHP
60 can be easily released in the environment since it is not covalently bound to plastic, and adheres to
61 organic particles (Zeng et al., 2008; Magdouli et al., 2013). As a result, DEHP is widely dispersed
62 in the environment *via* sewage systems, waste disposals and sprayings (Fromme et al., 2002; Porter
63 & Janz, 2003; Wang et al., 2011; Casals-Casas & Desvergne, 2011). Moreover, DEHP can be
64 transported over long distances. As the chemical degradation of DEHP is practically null *in natura*,
65 many – if not all - organisms can be in contact with this compound during their life (Wofford et al.,
66 1981).

67 Aquatic or terrestrial organisms are contaminated by direct exposure to DEHP, or by
68 ingestion of DEHP-contaminated food (Guehlstorf, 2004; Bar-El & Reifen, 2010). In vertebrates,
69 DEHP disturbs the levels of circulating hormones, as well as the hormone-receptor interaction and
70 the steroid signalling pathways (Wetherill et al., 2007; Stoker et al., 2008; Vanderberg et al., 2009).
71 These disruptions can lead to dysregulations of several processes including metabolic disruption
72 reported in goldfish or in rat cardiac cells (Jordan et al., 2012; Posnack et al., 2012).

73 In invertebrates, and more particularly in insects, the impact of DEHP has been poorly
74 investigated despite the crucial role of these animals in ecology, agronomy, medicine and economy
75 (deFur et al., 1999; Oehlmann & Schulte-Oehlmann, 2003). Moreover, most studies have focused
76 on the impact of EDCs on aquatic insects, such as *Chironomus riparius* (Planello et al., 2011;
77 Herrero et al. 2017), a common ecotoxicological model. There are few studies that have been
78 conducted on terrestrial insects. One study on the ant *Lasius niger* reported a reduction of egg-
79 laying rate in queens exposed to DEHP, together with an activation of their immune system
80 (Cuvillier-Hot et al., 2014). *Drosophila melanogaster* contaminated by DEHP exhibited a life span
81 reduction and a modulation of cholinergic transmission in their projection neurons (Ran et al.,
82 2012). The mode of action of DEHP in insects is still not yet well understood. It has been suggested
83 that DEHP can disrupt the concentration of circulating hormones, such as ecdysteroids, or interfere
84 with hormone receptors, leading to the activation of inappropriate steroid signalling pathway
85 (Hanioka et al., 2008, Planelló et al., 2011; Tarrant et al., 2011). Since these hormones control many
86 developmental processes, such as moulting, metamorphosis (Siaussat et al., 2008) and reproductive
87 physiology (Bigot et al., 2012), endocrine disruption could have important consequences.

88 Soils and water contaminations by DEHP in agricultural ecosystems are now reported and
89 can reach quite high concentrations: e.g. up to 300-500 µg/L for surface and underground water and
90 up to 30 mg/kg for agricultural soils (Zeng et al., 2008; Uren-Webster et al., 2010; Planello et al.,
91 2011; Wang et al., 2017). In sludge potentially used to fertilize agricultural soils, DEHP reaches
92 concentrations ranging from 150 to 600 mg/kg dry weight (dw) (Bergé et al., 2013), with
93 subsequent bioaccumulation into cultivated plants (EU, 2008; Sun et al., 2015). Several studies
94 highlighted DEHP accumulation in cultivated plants with a range of concentration going from 0.1 to
95 4150 µg of DEHP per gram of plant or leaf (i.e. dry weight) and from 26.5 to 362 µg of DEHP per
96 gram of peel and flesh of three fruits (He et al. 2016; Zhao et al. 2015; Wu et al. 2013; Fu & Du,
97 2011; Du et al. 2010; Du et al., 2006). Herbivorous insects are likely to ingest DEHP when feeding
98 on cultivated plants (Staples et al., 2010). In the present study, we therefore used the highly
99 polyphagous and widespread insect species, the Egyptian cotton leafworm *Spodoptera littoralis*
100 (Boisduval) (Lepidoptera: Noctuidae) as a model to investigate the effects of DEHP on the biology
101 and physiology of a terrestrial insect.

102 This study was designed in order to answer the following questions: (1) What are the
103 consequences of continuous ingestion of DEHP-contaminated food in terms of bioaccumulation in
104 *S. littoralis*? (2) What are the effects of this treatment on post-embryonic development? (3) Does

105 DEHP disrupt the molecular events that occur during the last larval stage (critical stage before the
106 passage to chrysalis)? (4) Considering the known effects of the EDCs, does DEHP exposure induce
107 effects at multiple levels (*e.g.* gene expression, metabolism, hormonal concentration) *in S.*
108 *littoralis*?

109 To address these questions, we first monitored the effects of DEHP ingestion on the post-
110 embryonic development of *S. littoralis* by analysing several key parameters, such as the duration of
111 larval stages, larval weight, and sex ratio. In parallel, we measured DEHP concentrations at all
112 stages of the life cycle. Then, we focused on the 5th, 6th and 7th days of the last instar, a crucial
113 period preceding the pupal stage and the metamorphosis. The expression levels of four nuclear
114 receptors involved in the ecdysteroid signalling pathway were measured: EcR, the 20-
115 hydroxyecdysone (20E) receptor; Ultraspiracle (USP), its heterodimeric partner and two early-late
116 genes, E75A/B and E75D. We also measured ecdysteroid hemolymphatic concentrations and drew
117 a physiological footprint of DEHP effects by running metabolomics assays.

118
119

120 **Material and methods**

121

122 **Insect rearing, DEHP treatment and study of the post-embryonic development**

123 *S. littoralis* larvae were reared on a semi-artificial diet (stored in glass jars at 4 °C prior to
124 use) from the third instar to pupation at 23° C, 60%-80% relative humidity, and 16:8h light:dark
125 cycle (Hinks & Byers, 1976). Six environmentally relevant concentrations (100pg; 1ng; 10ng;
126 100ng; 1µg and 10µg per gram of food) and three high concentrations (500µg, 5mg and 40 mg per
127 gram of food) of DEHP (PESTANAL® 36735, Sigma, France) were added to the diet. Control
128 larvae were reared on food containing only the solvent (0.5% pure Ethanol, absolute, PROLABO®,
129 VWR, France). Larval mass, food consumption and mortality rate were monitored daily. The
130 presence of a cephalic capsule was checked every day in order to determine the instar duration, and
131 the beginning of ecdysis. Male and female chrysalises were kept separately in order to determine
132 the pupal stage duration, the sex-ratio and the percentage of mortality.

133

134 **Measure of DEHP content**

135 *Sample Treatment*

136 It is important to realize DEHP is present in the environment. Thus, the DEHP treatments
137 administrated to the animals underestimate the real level of contamination. As a result, we estimated
138 the actual level of contamination. DEHP content was measured in several treated and control larvae,
139 pupae (from 4 to 6 days-old) and adults (2 days-old). Considering the atmospheric contamination in
140 our lab conditions, as described by [Lenoir et al. \(2012\)](#), we also measured the concentration in
141 control and contaminated food (100 pg/g, 1ng/g, 100ng/g, 10µg/g and 5mg/g) and realized a linear
142 regression analysis between theoretical applied concentrations and actual measured concentrations
143 in order to indicate directly in the figures the measured concentrations or estimated by the linear
144 regression (see results). Since the concentration measured in food for the 1ng/g condition was
145 similar to the control one, we subsequently performed the measurement of DEHP contents only for
146 the insects treated with 100ng, 10µg and 5 mg/g contaminated diet, and their controls (see results).
147 Before treatment, artificial diet and whole *S. littoralis* adult males and females were stocked at -18
148 °C, lyophilized and crushed in a mortar. Samples, 0.2g dw, were spiked with an internal standard
149 (IS) (DEHP-d4: 2µg) in a 40mL centrifuge glass tube overnight at 4°C. The next day, samples were
150 extracted with 15mL of hexane/acetone (50/50 [v/v]) in a Bransonic ultrasonic bath during 20 min
151 (US-EPA method 3550B for ultrasonic extraction 1996). Extracts were centrifuged (2 min at 4500
152 rpm), and supernatants were kept. This procedure was performed twice, and both extracts were
153 combined before concentration with EZ-2 system and finally under a nitrogen stream. The clean-up
154 procedure, derived from USEPA method 3620B (for phthalate ester and PCB fraction cleanup),
155 consisted of partitioning on a Florisil cartridge (Supelco [1 g]). After conditioning, loading extract
156 and washing with 10mL of hexane, DEHP was eluted with 10mL of hexane/acetone (90/10 [v/v]).

157 The extract was concentrated (400 μ L) under a nitrogen stream and deposited in injection vial before
158 analysis.

159

160 Analytical Conditions

161 Analyses were performed by gas chromatography–mass spectrometry (GC–MS) with a 7890
162 A gas chromatograph coupled to a 5975 A mass spectrometer (Agilent Technologies, Les Ulis,
163 France). Analytical conditions are summarized in Table S1.

164

165 Validation method

166 In our experiment, instrument detection limits (IDLs), *i.e* concentration of DEHP in the
167 standard solutions corresponding to a signal to noise ratio (SNR; peak to peak) of 3, was of 2pg
168 DEHP. Method detection limits (MDLs), corresponding to the concentration of spiked sample for a
169 SNR (peak to peak) of 9, was of 46ng DEHP/g (dw). Finally, recoveries for the entire procedure
170 were estimated by the determination of recovery rate (RR), which was 84% in our experiment.
171 Simultaneously to each set of analyses (n = 10), method blanks were performed.

172

173 RNA isolation and cDNA synthesis

174 Larvae were sampled on day 5, 6 and 7 of the last instar, and stored at -80 °C until total
175 RNAs extraction by TRIzol method (Invitrogen, Carlsbad, CA, USA) coupled to RNAeasy kit
176 extraction (Qiagen, USA). RNA quality was checked by spectrophotometry at 260nm and 280nm
177 (BioPhotometer, Eppendorf, Hamburg, Germany). Treatment with DNase I (Roche, USA) was
178 made in accordance with the manufacturer's instructions. Single-stranded cDNAs were synthesized
179 from 5 μ g of RNA with Superscript II reverse transcriptase (Invitrogen) using the manufacturer's
180 protocol. For each experimental condition, four to five biological replicates were performed.

181

182 PCR and qPCR

183 Five genes were first tested as putative housekeeping genes (*actin*, *ATPase*, *Rpl13*, *tubulin*
184 *and ubiquitin*) by Bestkeeper analysis (Pfaffl et al. 2004). *Rpl13* (GenBank FJ979921) was used as
185 the reference gene, as it had the most stable expression level among experimental groups. Forward
186 and reverse primers for housekeeping and candidate genes (*ECR*, *USP*, *E75A/B*, *E75D*)
187 (Supplementary data Table S2) were designed from EST library sequences using EPRIMER 3
188 software, as described in Bigot et al. (2012). Since the isoforms A and B of E75 have few amino
189 acid differences in their N-terminal parts, primers were designed in a common region, allowing the
190 accurate amplification of the two isoforms. PCR reactions were performed on the LightCycler480
191 Real-Time PCR Detection System (Roche Applied Science, France), adapted from Bigot et al.
192 (2012). Each reaction consisted of 5 μ L of Absolute Blue SYBR Green Fluor (Thermo Scientific,
193 Waltham, MA, USA), 4 μ L cDNA (6.25ng/ μ L) and 0.5 μ L of each primer (10 μ M). The PCR
194 program consisted of an initial step at 95 °C for 5 min, followed by 50 cycles, each comprising 10s
195 at 95 °C, 15s at 60 °C, and 15s at 72 °C. Under these conditions, a single and discrete peak was
196 detected for all primers tested, and all primers gave efficiencies of 90-100%. Each run included the
197 fivefold dilution series, the candidate genes, the reference gene, and negative controls. The average
198 C_t value of each triplicate reaction was used to normalize the candidate gene expression level to the
199 geometric mean of *Rpl13* level in Q-Gene (Simon, 2003).

200

201 Hemolymph collection

202 Larvae were sampled on day 5, 6 and 7 of the last instar. Hemolymph was collected by
203 piercing the dorsal cuticle and epidermis with a sterile scalpel blade. The drop of hemolymph
204 obtained from 1 to 4 individuals (for a total of 40 \pm 10 μ L) was stored at -20 °C with 250 μ L of
205 methanol (purity 99.9%, MERCK, France) for ecdysteroid titration, or at -20 °C with 400 μ L of
206 ethanol (purity 99.9%, MERCK, France) for metabolomic profiling.

207

208

209 **Ecdysteroid titration**

210 Six to fifteen biological replicates were prepared for each experimental condition. After a
211 centrifugation at 9,500 g for 10 min, the supernatant was collected and dried under vacuum using a
212 SpeedVac Concentrator (Eppendorf, France). Samples were dissolved in a buffer for an Enzyme
213 Immuno Assay (EIA) adapted from [Porcheron et al. \(1989\)](#). The EIA titration was made using a
214 polyclonal anti-20E antiserum L2 (dilution 1:62,500) and a 2-Succinyl-20-hydroxyecdysone
215 coupled to peroxidase (dilution 1:100,000). O-Phenylenediamine, OPD and hydrogen peroxide
216 solution tablets (OPD, Sigma, France) were used for the chromogenic reaction and optical density
217 (OD) was measured at 450 nm. Calibration curves were produced for L2 antiserum using 20E
218 (range from 16 - 2000 fmol; gift from René Lafont, UPMC, Paris, France) and data were expressed
219 as pg 20E equivalent / μ L of hemolymph.

220

221 **Metabolomic profiling**

222 Ten biological replicates were prepared for each experimental condition, as described in
223 [Khodayari et al. \(2013\)](#). The samples were homogenized in 600 μ L of methanol-chloroform (2:1)
224 using a bead-beating device (RetschTM MM301, RetschGmbH, Haan, Germany). A volume of 400
225 μ L of ice-cold ultrapure water was added to each sample, before centrifugation at 4,000 g for 10
226 min at 4 °C. Then, aliquots (300 μ L) of the upper aqueous phase containing polar metabolites were
227 transferred to microtubes and vacuum-dried. Following derivatization (see [Khodayari et al., 2013](#)
228 for the detailed procedure), metabolites were analyzed by gas chromatography-mass spectrometry
229 (GC-MS), which included a CTC CombiPal autosampler (CTC Analytics AG, Zwingen,
230 Switzerland), a Trace GC Ultra chromatograph, and a Trace DSQII quadrupole mass spectrometer
231 (Thermo Fischer Scientific Inc., Waltham, MA, USA) ([Khodayari et al., 2013](#)). Peaks were
232 annotated using both mass spectra (two specific ions), and retention times. Calibration curves were
233 set using standards consisting of 57 pure reference compounds most often quantified in insects with
234 this equipment. Metabolite levels were quantified using XCalibur v2.0.7 software (Thermo Fisher
235 Scientific Inc., Waltham, MA, USA).

236

237 **Statistical analysis**

238 Statistical analyses were performed using R 3.2.2 and R Studio 1.0 (R Core Team, 2015),
239 JMP (SAS institute, France) and XLstat software (Addinsoft, France). Shapiro-Wilk test was used
240 to check the normality of the data, and a Bartlett's test was used for testing the homogeneity of the
241 variance. Sex-ratio was analyzed by comparison of proportions with a bilateral test (Monte Carlo
242 method). Larval body mass changes and food consumption were analyzed with the Dunnet's Test
243 using control animals as a reference group. Durations of larval and pupal developmental stages
244 were analyzed with one-way ANOVA. Mortality rates during larval and pupal stages were analyzed
245 with a logistic regression test. Hemolymphatic concentrations of ecdysteroids and DEHP were
246 compared using a Wilcoxon sum of rank test. qPCR results were analyzed with an ANOVA and
247 Student t test. Metabolomic results were analyzed using ANOVA and Student t test for data
248 respecting the conditions for parametric tests (Variance and normality) or using a Wilcoxon test for
249 the others. In all cases, p-values lower than 5% were considered significant.

250

251

252 **Results**

253

254 **DEHP contents in diet**

255 Control diet contained an average of 392 ± 125 ng of DEHP per g (fresh weight) (Figure 1A).
256 This value was similar to the diet spiked with DEHP at 100 pg/g and 1ng/g of food (i.e. 443 ± 138
257 and 676 ± 160 ng/g) and this amount would be considered as a threshold of atmospheric
258 contamination. Concentrations of DEHP were significantly higher for diets treated with 100 ng, 10
259 μ g and lower for diet treated with 5 mg DEHP/g (Figure 1; Wilcoxon test, $P < 0.01$), with values of
260 1.1 ± 0.225 μ g, 19.7 ± 1.9 μ g, and 4.3 ± 0.229 mg DEHP/g, respectively. These measured values were

261 thus used as references for the rest of the study, instead of the theoretical ones (Figure 1B). The
262 lacking values (i.e. for the theoretical values: 1µg, 500µg and 40 mg) were determined by a linear
263 regression analysis using the measured values (Figure 1B).

264 For the control, 1.1µg and 19.7µg/g conditions, we observed that DEHP contents were
265 higher in pupae and adults in comparison to larval stages (Figure 2, statistical results not
266 represented, Wilcoxon test, $P < 0.05$). Conversely, a significant higher DEHP content was observed
267 in L5 and L6 larval instar fed with the highest concentration (i.e. 4.3 mg/g) in comparison to pupal
268 and adult stage (Figure 2, statistical results not represented, Wilcoxon test, $P < 0.05$).

269 The focus on each stage highlighted that we observed most of the time a significant higher
270 DEHP content in all stages corresponding to the 4.3 mg/g condition in comparison to control and
271 others conditions (Figure 2, Wilcoxon test, $P < 0.05$, see first uppercase letters). But we noted an
272 exception for the female pupae for which the DEHP content for the 4.3 mg/g condition was not
273 significantly different to control (Figure 2, Wilcoxon test, $P < 0.05$, see first uppercase letters). Most
274 of the time the DEHP content measured in treated groups was similar to control (i.e. no statistical
275 difference) or higher to control content (Figure 2, Wilcoxon test, $P < 0.05$, see first uppercase letters).
276 Female pupae presented an exception since the DEHP content in 1.1 µg female pupae was
277 significantly lower to control (Figure 2, Wilcoxon test, $P < 0.05$, see first uppercase letters).

278 When focusing only on the larval stage, a specific pattern was observed with a significant
279 decrease of DEHP concentration between the L6 and L7 for all DEHP conditions (Figure 2,
280 Wilcoxon test, $P < 0.05$, see second lowercase letters). Differences in DEHP concentrations appeared
281 also between the males and the females. Thus, control female pupae have a significant higher
282 DEHP content in comparison to males (Figure 2, Wilcoxon test, $P < 0.05$, see second lowercase
283 letters), whereas female pupae treated with 1.1 µg/g have a significant lower DEHP content in
284 comparison to males of the same DEHP concentration (Figure 2, Wilcoxon test, $P < 0.05$, see second
285 lowercase letters). A similar pattern was observed for adults with a higher DEHP content in control
286 females and lower concentrations for all treated females in comparison to males (Figure 2,
287 Wilcoxon test, $P < 0.05$, see second lowercase letters). Thus, our results highlighted that management
288 of DEHP appears to be different in males and females.

289

290 **Impact of DEHP on post-embryonic development**

291 The highest concentration of DEHP induced a high mortality from the fourth to the seventh
292 instar, leading to the death of all larvae at the time of the pupation (Figure 3; logistic regression test,
293 $P < 0.001$ for all larval stages). High mortality was also observed for larvae fed with DEHP at 4.3
294 mg/g, with a cumulative mortality of 30% at L7 and 36% at pupal stage (Figure 3; logistic
295 regression test, $P < 0.01$ for all larval stages).

296 We then analyzed the effects of DEHP on post-embryonic development time by exposing
297 larvae to various DEHP concentrations in their diet. The highest DEHP concentration increased the
298 duration of the fourth, fifth and sixth instars (L4, L5, L6) (Figure 4; ANOVA $F_{10,464}=1.02$,
299 $P < 0.0425$ with Tukey HSD test $P < 0.05$ to 0.001 for L4; ANOVA $F_{10,468}=1.996$, $P < 0.0321$ with
300 Tukey HSD test $P < 0.05$ to 0.001 for L5; ANOVA $F_{10,477}=15.52$, $P < 2e-16$ with Tukey HSD test
301 $P < 0.05$ to 0.001 for L6). The duration of the pupal stage was longer for the larvae treated with
302 19.7 µg and 447.2 µg of DEHP per g of diet, but without changes in larval stages (Figure 4;
303 ANOVA $F_{10,519}=24.68$, $P < 2e-16$ with Tukey HSD test $P < 0.05$ to 0.001). Globally, by
304 cumulating durations of larval and pupal stages (Figure 4, histogram named "Total"), we observed a
305 significant increase in the postembryonic development time for larvae exposed to 3.2 µg, 19.7 µg,
306 447.2 µg and 4.3 mg/g of DEHP (Figure 4). Finally, sex ratio was modified at 3.2 µg DEHP/g
307 (Figure 5; Bilateral test with Monte Carlo method, $P < 0.05$ to 0.01): the percentage of females was
308 increased (56.2 ± 4.8 %) in comparison to control pupae (48.5 ± 3.7 %).

309 DEHP treatment had no effect on larval mass gain of the surviving larvae, as compared with
310 control larvae, except for larvae that were exposed to the highest DEHP concentration (19.5 mg/g)
311 (Figure 6). In this latter group, the larvae grew more slowly, reaching a body mass two times lower
312 than their control relatives at day 12 (Figure 6A, Dunnett's Test, $P < 0.05$ to 0.001). At this DEHP

313 concentration, the surviving larvae reached the same body mass than the controls just before
314 pupation. From the middle of the 6th larval instar till pupation, the increase of food consumption
315 was slower for larvae grown on the 19.5 mg/g DEHP diet, as compared with control larvae (Figure
316 6B, Dunnett's Test, $P < 0.05$ to 0.001). For all other experimental conditions with DEHP, the
317 increase of food consumption during the last larval instar was higher than in control larvae (Figure
318 6B, Dunnett's Test, $P < 0.001$ to 0.05).

319 **Hemolymphatic concentrations of ecdysteroids in larvae**

320 Larvae treated with DEHP at $1.1 \mu\text{g/g}$, $19.7 \mu\text{g/g}$ and 4.3 mg/g and control larvae were
321 sampled at day 5, 6 or 7 during the last instar in order to measure their ecdysteroid titration. A peak
322 of ecdysteroids is usually observed during the last instar of Lepidoptera, before the onset of pupal
323 stage and metamorphosis. Our results showed that this peak can be observed at day 6 of L7 in *S.*
324 *littoralis* larvae for all conditions (Figure 7). At day 7, we observed that ecdysteroid concentrations
325 were higher in larvae fed with the highest dose of DEHP than in those fed with the lowest one
326 (Figure 7, Wilcoxon's test, $P < 0.05$).

327 **Expression levels of nuclear receptors during the last larval instar (L7)**

328
329 A DEHP-concentration dependent decrease in EcR-B gene transcription was observed at day
330 6. This decrease was significantly different for the highest concentration of DEHP (*i.e.* 4.3 mg/g)
331 (Figure 8, Student t test, $P < 0.05$). USP gene expression was lower than in controls at days 5 and 6
332 for concentrations of DEHP in the diet of $19.7 \mu\text{g/g}$ and 4.3 mg/g , and at day 7 for $19.7 \mu\text{g/g}$
333 (Figure 8, Student t test, $P < 0.05$ to 0.001). No difference was observed in E75A/B and E75D gene
334 expression levels at days 5 and 6, while E75D gene expression was lower at day 7 in larvae fed on
335 diet with $1.1 \mu\text{g}$ DEHP, but higher at 4.3 mg/g (Student t test, $P < 0.05$ for all comparisons).

336 **Metabolomic profiling**

337
338 Forty-four metabolites were quantified from the hemolymph of *S. littoralis* larvae. A
339 metabolic transition was observed from the 5th to the 7th day, with increased concentrations of a
340 significant number of metabolites involved in glycolysis (glucose, glucose-6P), TCA cycle (citrate,
341 fumarate, malate, succinate), pentose phosphate pathway (adonitol, ribose, sorbitol, xylitol), fatty
342 acid biosynthesis (galacturonic acid, glycerol, inositol), gluconeogenesis (glucose and lactate),
343 amino acid production (alanine, asparagine, aspartate, glutamate, isoleucine, leucine, lysine,
344 phenylalanine, threonine, valine) and others metabolic processes (gluconolactone, pipercolic acid,
345 putrescine, spermidine) (Figure 9 and supplementary data Table S3 for details). Some metabolites
346 as GABA, glycerol-3P, glycine, proline, serine and trehalose had a different pattern with a decrease
347 of hemolymphatic concentrations. Valine displayed also a unique pattern characterized by a
348 transient decrease from day 5 to 6, followed by an increase to higher concentrations at day 7. The
349 day-by-day comparison between DEHP treated larvae with control larvae highlighted effects of
350 DEHP on the amounts of twenty metabolites (Table 1). No difference was observed between
351 control and the DEHP-treated larvae at day 5. At the 6th day, seventeen metabolites had a lower
352 concentration in larvae fed with $1.1 \mu\text{g/g}$ of DEHP in comparison to control larvae (Table 1,
353 Student t test, $P < 0.05$ to 0.01). Several metabolites had similar profiles at the 6th day for other 19.7
354 $\mu\text{g/g}$ of DEHP. Lower concentrations of alanine, citric acid, glycerol, putrescine, serine and valine
355 in larvae fed with $19.7 \mu\text{g/g}$ of DEHP were also measured. Energetic disruption is induced by
356 DEHP since, on the seventeen metabolites, four are involved in TCA cycle, two in glycolysis, one
357 in gluconeogenesis and three in pentose phosphate pathway. Amino acids synthesis seemed the
358 second main disrupted metabolic mechanism with the decrease of the concentration of eight amino
359 acids. At the 7th day, lactic acid presented a lower concentration in larvae fed with 4.3 mg/g , while
360 at the same treatment trehalose concentration increased. Whereas the concentration of some
361 metabolites was modified only for one DEHP treatment (*i.e.* erythritol, galactose, GABA, inositol,
362 malic acid, phenylalanine, pipercolic acid, xylitol), the concentration of several metabolites was
363

364 similarly modified for two DEHP treatments, (*i.e.* alanine, citric acid, glyceric acid, glycerol,
365 putrescine and valine).

366

367 Discussion

368 *Environmental contamination and bioaccumulation of DEHP in S. littoralis*

369 In this work, we aimed at investigating the effects of DEHP on the physiology of a terrestrial
370 insect, by growing *S. littoralis* on DEHP-contaminated food. We first monitored the amount of
371 DEHP present in control diet (*i.e.* 392±125 ng of DEHP per g of diet fresh weight) and found that it
372 was quite similar to the concentration found in the contaminated diet initially prepared to reach a
373 theoretical concentration of 1ng/g of DEHP. This result shows that the environmental pollution
374 should be measured to determine the basic level of contaminant during experimental procedure.

375 Our result also highlighted the challenge to measure DEHP in complex matrix. Indeed, we
376 can observe that the lower the considered theoretical DEHP concentration, the higher is the
377 difference between the theoretical and measured concentrations. After removing the basal
378 contamination found in the controls (*i.e.* 392 ng/g), we observed that the measured levels for the
379 two lowest DEHP concentrations were 510 and 284 fold higher than theoretical, from 1.93 to 7.1
380 fold higher for the intermediate DEHP concentrations and closer to theoretical with the highest
381 DEHP concentrations. We also noted that the SEM for the measured concentrations is increasing in
382 the same manner for the lowest DEHP concentration suggesting that there is a higher variability of
383 results when we are measuring the DEHP concentrations in the food with the lowest concentrations.
384 These results are consistent with the report of comparative studies that highlighted that the current
385 technics used for DEHP measurement have different selectivity/specificity, precision, sensitivity
386 (evaluation of LOD and LOQ) as well as various problems of repeatability and matrix interference
387 (Moldoveanu and Yerabolu, 2018). Moreover, matrix interference is usually one of the most
388 important problems that researchers have to face when quantifying trace compounds of phthalates
389 in complicated matrix samples (Snell, 1993; Ye et al, 2009). These interferences could lead to
390 problem of DEHP detection or to differences between theoretical and measured concentrations.
391 Even if these interferences do not call into question the results obtained on the development and the
392 physiology of the insects, our results highlighted the need to quantify the atmospheric
393 contamination but also the impact of matrix on DEHP measurement.

394 When fed with treated diet, larvae progressively accumulated DEHP, then the level of
395 DEHP decreased during the last larval instars. This decrease was particularly spectacular for larvae
396 fed with the higher dose (4.3 mg/g) and it continues until pupation, to reach DEHP levels that were
397 closer to the levels observed for the other conditions. This decrease in DEHP content at the end of
398 the larval stage may be explained by the developmental processes that are taking place during this
399 time. Indeed, in order to prepare the pupal molt and metamorphosis, the larvae in the last days of
400 the last instar (*i.e.* period of prepupae formation) are starting starvation, shrinking in size and
401 purging their digestive system in order to eliminate the last metabolic wastes (Hu et al. 2016).
402 Moreover, we already highlighted the expression of several enzymes of detoxification in the larvae
403 of *S. littoralis* (Durand et al. 2010; Pottier et al., 2012) suggesting abilities of this insect to detoxify
404 xenobiotic compounds including DEHP.

405 A significant higher DEHP concentration in chrysalid and adults was observed for all
406 treatments including control in comparison to larval concentration. This increase could be partly
407 explained by the reduction of weight from last larval instar (average of 0.41 mg of fresh weight per
408 larvae) to chrysalid stage (average of 0.258 mg and 0.264 mg of fresh weight for males and females
409 chrysalids respectively). The loss of weight after desiccation of insects for DEHP measurement is
410 also a factor of difference. Thus, a 2.8-fold reduction of weight was observed in larvae whereas a
411 4.3 or 4.47-fold reduction was observed in males and females chrysalids respectively. But these two
412 factors of amplification could not explain alone the important increase of concentration and a
413 passive atmospheric contamination during chrysalid stage could be considered. Chrysalis is an
414 immobile stage, which has very low exchanges with the environment due to a high impermeability
415 of cuticle. At the exception of gaseous exchanges (*i.e.* O₂ and CO₂), a chrysalis is living on its stock

416 until adult emergence (Basson & Terblanche, 2010). Thus, in comparison to larvae that can detoxify
417 and eliminate DEHP, pupae do not have the possibility to perform such kind of physiological
418 processes. Larvae of the *Spodoptera* gender have important detoxification abilities. Thus, two
419 studies have compared the differences in the expression of detoxification genes during post-
420 embryonic development (Huang et al., 2011; Wand et al., 2015). They thus highlighted that a large
421 number of enzymes were expressed and often at high levels in larvae compared to other stages,
422 allowing them to metabolize xenobiotic compounds and plant allelochemicals. This important
423 expression of detoxification genes in *Spodoptera* larvae could also explain this important decrease
424 of DEHP in our study. Adults seemed to be able to restart detoxification of DEHP as they present
425 lower DEHP compared to chrysalises (Wilcoxon's test, $P < 0.05$). Interestingly, this phenomenon is
426 not general since we observed a difference in the concentration between males and females for
427 similar DEHP conditions. This result showed that there is probably a difference in the detoxification
428 processes between the two sexes. Even if there is to date no evidence of gender difference in
429 detoxification of DEHP in insects, several articles already highlighted it for other pollutants (Occai
430 et al., 2018).

431

432 *Effect of DEHP on post-embryonic development*

433 We observed in this study for the first time the effects of DEHP ingestion on the post-
434 embryonic development of a terrestrial insect. We highlighted by this study different disruptive
435 effects of DEHP on *S. littoralis*. However, DEHP is known to rapidly degrade to MEHP and this
436 metabolite is also known to induce various toxic effects in exposed organisms (Kalo & Roth, 2017).
437 Thus, although we did not measure MEHP concentrations in this study, it is highly likely that the
438 observed disturbances are due to DEHP and MEHP.

439 The two highest non-environmentally relevant concentrations tested here increased
440 significantly the mortality of larvae and pupae, whereas the other doses were not lethal. The highest
441 DEHP dose also induced a reduction of larval growth. This decrease may partially result from the
442 reduction of larval food consumption and/or from a toxic effect of DEHP on metabolism. In a
443 recent work, Cuvilliers-Hot et al. (2014) demonstrated that workers of the *Lasius niger* ant can
444 detect and avoid food contaminated by environmentally-relevant doses of DEHP. However, this
445 behavior was not observed for *S. littoralis* larvae fed on all the other DEHP conditions as their food
446 consumption was on contrary increased, while their body mass remained similar to that of control
447 larvae. In vertebrates, several deleterious effects of DEHP have been reported, on gut or digestive
448 glands, with induction of tumors (Chen et al. 2017), genotoxicity (Kitamoto et al., 2015), disruption
449 of cholesterol homeostasis, deregulation of the inflammatory response, and acceleration of
450 atherosclerosis (Zhao et al. 2016). As a result, DEHP may have induced digestive problems to *S.*
451 *littoralis* larvae such as malabsorption or altered digestion, which could have been compensated by
452 increased food intake. This increase could be also explained by the DEHP detoxification leading to
453 an increase of energetic needs in exposed insects. On the contrary, exposure to environmental doses
454 of DEHP led to an increase of female body mass in *Chironomus riparius*, and to increased larval
455 body mass in *Chironomus tentans* (Kwak & Lee, 2005; Lee et al., 2006).

456 DEHP treatments also altered the duration of the life cycle. Longer larval or pupal stages
457 were observed for intermediate (*i.e.* 19.7 μg and 442 μg) and high DEHP concentrations (*i.e.* 4.3
458 mg and 19.5 mg/g) leading globally to a delay in adult emergence after exposure to concentrations
459 higher than 1.1 $\mu\text{g/g}$ of DEHP. In crustaceans, several studies have shown that exposure to DEHP
460 disrupts the timing of development and molting without induction of mortality (Zou and Fingerman,
461 1997; Billingham et al., 2001; Marcial et al., 2003). For instance, the survival of the estuarine
462 copepod *Eurytemora affinis* (nauplius stage) was unaffected after ten days of DEHP exposure, but
463 the animals remained in the nauplius stage, or reached the copepodid stage later than controls
464 (Forget-Leray et al., 2005). However, in the marine copepod *Calanus helgolandicus* a prolonged
465 exposure (*i.e.* nine days) to DEHP did not decrease survival (Cole et al., 2015) and similarly, in the
466 springtail *Folsomia fimetaria* (Jensen et al., 2001), DEHP treatment had no effect on survival,
467 reproduction, growth, or molting of juveniles. However, there are some exceptions, with for

468 example the high sensitivity of the copepod *Parvocalanus crassirostris nauplii* to DEHP, with
469 significantly higher mortality rates after 48h as compared to control, even at very low
470 environmental concentrations (Heindler et al., 2017).

471 The developmental events that take place during the last larval stage depend on the peak of
472 20E. Our results highlight that the peak of 20E in *S. littoralis* is at day 6. Following the peak, a
473 decrease of the 20E concentration should be observed, as well as the induction of molecular
474 signaling pathways including nuclear receptor expression in various targeted tissues. All these
475 events trigger the prepupal and pupal molts. Any disruption of these processes could lead to
476 developmental disturbances, as we were able to see in our study. Significant changes in ecdysteroid
477 concentrations were indeed observed in L7 larvae fed on DEHP-enriched diet at day 7, in parallel to
478 modifications of the expression levels of several nuclear receptors (*i.e.* EcRB, USP and E75D)
479 during this last instar. We observed a decrease of *EcRB* expression at day 6 in larvae exposed to 4.3
480 mg/g of DEHP and of *USP* gene expression during three days (5 to 7) in larvae exposed to 19.7 μ g
481 and 4.3 mg/g of DEHP. This finding, consistent with the study of Herrero et al. (2017), suggests
482 that DEHP could function as an ecdysone antagonist. Even if feedback regulations of the early and
483 early-late genes on their own expression and on the expressions of *EcR* and *USP* can take place in
484 the ecdysteroid pathways (Ashburner, 1974; Huet et al., 1995), DEHP appeared to induce a
485 downregulation of *EcR* and *USP* genes in larvae of *S. littoralis* fed with 19.7 μ g and 4.3 mg/g of
486 DEHP. On the contrary, at day 7 *E75D* expression appeared to be increased at high dose but also
487 significantly reduced at lower dose (1.1 μ g DEHP/g), showing that DEHP can differently disrupt
488 endocrine pathway depending on the dose.

489 At the 7th day, L7 larvae exposed to 4.3 mg/g DEHP had a higher hemolymphatic
490 concentration of ecdysteroids and a higher level of E75D expression than the controls; whereas the
491 larvae exposed to the lowest dose (*i.e.* 1.1 μ g/g) had a lower level of circulating hormone and of
492 E75D expression. In vertebrates, several studies highlighted that DEHP, can probably modulate the
493 level of circulating hormones by disrupting the activity of enzymes involved in the synthesis of
494 endogenous steroids and their metabolism (Thibaut & Porte, 2004). Even if down- and up-
495 regulations are usually taking place in the ecdysteroid signaling pathway, an increase of circulating
496 ecdysteroids generally induced an increase of nuclear receptor expression (Bigot et al., 2012; Jing et
497 al., 2016). Moreover, the chemical structure of DEHP, close to other endocrine disruptors as
498 DiethylPhthalate (DEP), may probably permit its binding to ecdysteroid receptors and disrupt
499 activation of the nuclear signaling pathway (Dinan et al., 2001). Our results showed both the effect
500 of DEHP exposure on hemolymphatic ecdysteroid concentrations and expression level of nuclear
501 receptors, bringing out the endocrine disruption nature of DEHP in *S. littoralis*. These variations
502 may contribute in the modification of the duration of the larval and pupal stages observed for some
503 DEHP treatments.

504 We also noted a significant imbalance in the male *versus* female ratio, showing a slight
505 feminization of the population for one treatment. If modification of sex ratio by DEHP was already
506 reported in vertebrates (Tanaka, 2003; Xi et al., 2011; Hou et al., 2015), there are very few studies
507 on its effect (or of other plastic products) on the sex ratio in arthropods. Kim and Lee (2004)
508 observed a modification of hatching rates of F1 egg ropes from DEHP exposed adult *C. riparius*,
509 without effects on sex-ratio. In particular, Forget-Leray et al. (2005) highlighted a feminization of
510 the copepod *E. affinis* after an exposure to a mix of estradiol and DEHP. Conversely, bisphenol A
511 (BPA) induced a feminization in *C. riparius* (Lee & Choi, 2007). It is generally accepted that sex
512 determinism in insects is predominantly genetic, and numerous studies showed the importance of
513 gene expression or epigenetic regulation in sex determination (Kan et al., 2017; Mine et al., 2017).
514 Thus, the sex ratio disruption in *S. littoralis* may more probably result from an endocrine disruption
515 of these processes rather than to hemolymphatic ecdysteroid variations.

516 The metabolomic profiling performed here highlighted a progressive change of the
517 metabolic phenotype during the last larval instar of *S. littoralis*. This transition may have mirrored
518 hemolymphatic changes of ecdysteroid concentrations, which peak induces the next molt and the
519 transition to the pupa, which has a quite different metabolism than larvae. The rising concentrations

520 of a large number of metabolites from the 5th to the 7th day reflected the increased activity of the
521 pentose phosphate pathway, the fatty acids biosynthesis, gluconeogenesis, glycolysis, TCA cycle
522 and amino acids' pathways. The general increase in biosynthesis activity may prepare larvae to the
523 pupal stage, during which insect has high energetic requirements linked to metamorphosis. At the
524 6th and 7th day, for the doses of 1.1 µg and 19.7 µg of DEHP, we observed a decrease in the
525 concentrations of several metabolites mainly involved in energetic production and amino acids
526 production. It is likely that the energetic costs of DEHP/MEHP detoxification and elimination could
527 impact the metabolic activities of the prepupae. We should also consider that DEHP/MEHP can
528 have a direct inhibitory effect on enzyme activity or expression as already demonstrated in the
529 literature (Wang et al., 2018).

530 More generally, there also seems to be a shunt in the energetic metabolism since the
531 decrease of several metabolites (*i.e.* alanine, serine, threonine, leucine, phenylalanine) coupled to a
532 decrease of the TCA cycle (*i.e.* citrate, fumarate, malate, succinate) seems to benefit the production
533 of glucose through gluconeogenesis. This decrease of metabolites involved in various energetic
534 pathways could be potentially the expression of this negative biological cost of DEHP on
535 developmental processes. Our results are consistent with the negative effect of DEHP on energetic
536 production and more particularly on TCA cycle already reported in vertebrates (Chiang et al.,
537 2016). At the highest dose of DEHP (*i.e.* 4.3 mg/g), the stress seemed to be very important (*i.e.*
538 mortality induction for some individuals) and no significant effect was observed on metabolites'
539 concentrations at the exception of the lower quantity of lactic acid indicating a probable reduction
540 of the gluconeogenesis process and an increase of trehalose, which is involved generally in cellular
541 protection. Levels of trehalose, the prominent 'blood sugar' in most insect species (Thomson,
542 2003), have already been found to increase in *Bombyx mori* and *S. litura* following exposure to
543 sublethal doses of various pesticides (Nath, 2003; Etebari et al., 2007; Zhu et al., 2012, Dewar et al.,
544 2015), suggesting that this sugar serves a protective role for proteins and cellular membranes from
545 inactivation and/or denaturation (Elbein et al., 2003).

546

547 Conclusion

548 We showed here that DEHP acts as an endocrine disruptor in *S. littoralis*, by inducing
549 variations in ecdysteroid and nuclear receptor levels. However, the effects were dependent on
550 DEHP doses. The highest concentrations, currently not observed in the environment, had stressful
551 consequences, by inducing mortality, physiological and growth problems. The intermediate doses,
552 found in environment, did not induce marked effects on *S. littoralis*. Further investigations have to
553 be done in order to study the abilities of larvae to detoxify or eliminate DEHP. The disruption of
554 metabolite quantities could potentially be linked to the increase of the pupal period duration since
555 not enough metabolites were stocked before this stage. Considering all these results, it would be
556 very interesting to study the effect of DEHP on later processes such as reproduction and cross
557 generational effects.

558

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 790
 791

792 **Legends**

793
 794 Figure 1. DEHP concentrations in control and contaminated diet (in ng of DEHP per gram of
 795 fresh food). (A) Quantity of DEHP measured in control and contaminated diet in ng per g of
 796 food (fresh weight). Each bar represents the mean of values with representation of SEM (n=6 to
 797 11 for each condition). (B) Correspondence between theoretical and measured^(*) or estimated^(e)
 798 concentrations in contaminated diet. Asterisks indicate significant differences among means in
 799 comparison to controls (* P<0.05; ** P<0.01; *** P<0.001).
 800

801 Figure 2. DEHP concentrations during development of *S. littoralis*. Concentrations in ng of
 802 DEHP per gram of fresh weight found in larval stages (*i.e.* L5, L6 or L7), in males and females
 803 during the pupal and adult stages (n=3-4 biological replicates for each condition corresponding
 804 to a pool of 3 to 10 individuals). For each condition and stage, the mean value ± SEM was
 805 indicated. For statistical analysis, significant difference is indicated by a different letter. The
 806 first uppercase letter, X in (X/y), is for the statistical comparison by focusing on one specific
 807 stage between the different DEHP conditions (*i.e.* control, 1.1 µg, 19.7 µg and 4.3 mg/g). The
 808 second lowercase letter, y in (X/y), is for the statistical comparison made for a similar DEHP (or
 809 control) condition between only L5, L6, L7 stages or between only male (C♂) and female (C♀)
 810 chrysalises, or finally between only adult males (♂) and females (♀).
 811

812 Figure 3. Effect of DEHP on the mortality of larval and pupal stages. Cumulated percentages of
 813 mortality were compared between larvae treated with DEHP and control larvae (n=35 for each
 814 conditions). Asterisks indicate significant differences among means in comparison to controls (*
 815 P<0.05; ** P<0.01; *** P<0.001).
 816

817 Figure 4. Effect of DEHP on post-embryonic development time. The durations (in days) of
 818 larval (*i.e.* L4, L5, L6, and L7) and pupal stages observed for DEHP-contaminated larvae were
 819 compared to the control conditions (n=35 for each conditions). The complete development time
 820 from L4 up to adult emergence is also represented (“Total”). Different letters indicate a
 821 significant difference between controls and treated individuals of a same stage.
 822

823 Figure 5. Effect of DEHP on the sex ratio repartition at pupal stage. The percentages of male
 824 and female pupae obtained from larvae treated with DEHP by food ingestion (from 1.1µg/g to
 825 19.5 mg/g) were compared to the percentage of controls (n=72 to 100). Asterisks indicate
 826 significant differences among means in comparison to controls (* P<0.05; ** P<0.01; ***
 827 P<0.001).
 828

829 Figure 6. Effect of DEHP on larval weight (**A**) and food consumption (**B**) after continuous
 830 ingestion of food contaminated with various concentrations of DEHP. Weights of larvae and
 831 food were measured at each molt for L4, L5 and L6 and daily during the last larval instar (L7).
 832 Each point represents the mean value (n=35 for each conditions). The bar under the horizontal
 833 axis indicates the corresponding larval instar. Asterisks indicate significant differences among
 834 means in comparison to controls (* P<0.05; ** P<0.01; *** P<0.001).
 835

836 Figure 7. Quantification of hemolymphatic concentration of ecdysteroid (in fmol 20E eq/µL) in
 837 contaminated or control larvae at the 5th, 6th and 7th day of the last larval instar (n=7 to 12).
 838 Ecdysteroid concentrations were quantified by EIA. Bars with asterisks indicate significant
 839 differences among means in comparison to controls (* P<0.05; ** P<0.01; *** P<0.001).
 840

841 Figure 8. Normalized expression of nuclear receptor (EcRB, USP, E75D, E75A/B) in
 842 contaminated or control larvae at the 5th, 6th and 7th day of the last larval instar (n=6 for each
 843 condition). Expression levels were quantified by quantitative PCR. Bars with asterisks indicate
 844 significant differences among means in comparison to controls (* P<0.05; ** P<0.01; ***
 845 P<0.001).
 846

847 Figure 9. Schematic representation of the metabolic pathways in the last instar of control larvae
 848 with variation of metabolite concentration between the 5th, 6th and 7th day of this larval stage.
 849 Hemolymph of treated or control larvae were collected, pooled and analyzed by GC-MS.
 850 Metabolites in bold were detected and quantified in the hemolymph. A graphical representation
 851 in grey of the concentrations of these metabolites was added in order to show the variation from
 852 5th to 7th day of the last larval instar. Original graphs can be found in supplementary data
 853 (supplementary data figure S1). Metabolites surrounded by a black frame have a significant
 854 decrease of concentration in comparison to control during the 6th day of the last instar (see
 855 results Table 1).
 856

857 Table 1. Comparison of metabolites concentrations in the hemolymph of DEHP treated larvae
 858 during the 5th, 6th and 7th days of the last larval instar in comparison to control larvae. Only the
 859 metabolites showing a significant variation in comparison to control were presented in this
 860 table. “>” and “<” indicate that the measured concentration of the corresponding metabolite is
 861 respectively superior or inferior to control concentrations. Asterisks indicate significant
 862 differences among means in comparison to controls (* P<0.05; ** P<0.01; *** P<0.001).
 863

864 Table S1. Analytical conditions used for determination of DEHP content in *S. littoralis* adult
 865 males and females by GC-MS. IS: internal standard
 866

867 Table S2. Sequences of the primers used for RTqPCR amplification in *Spodoptera littoralis*
 868 larvae. F: forward primer; R: reverse primer

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Table S3 Minimum and maximum metabolite concentrations and non-detected metabolites on the three days of GC-MS analysis in treated and control larvae. Concentrations were expressed in nmoles / μ L of hemolymph.

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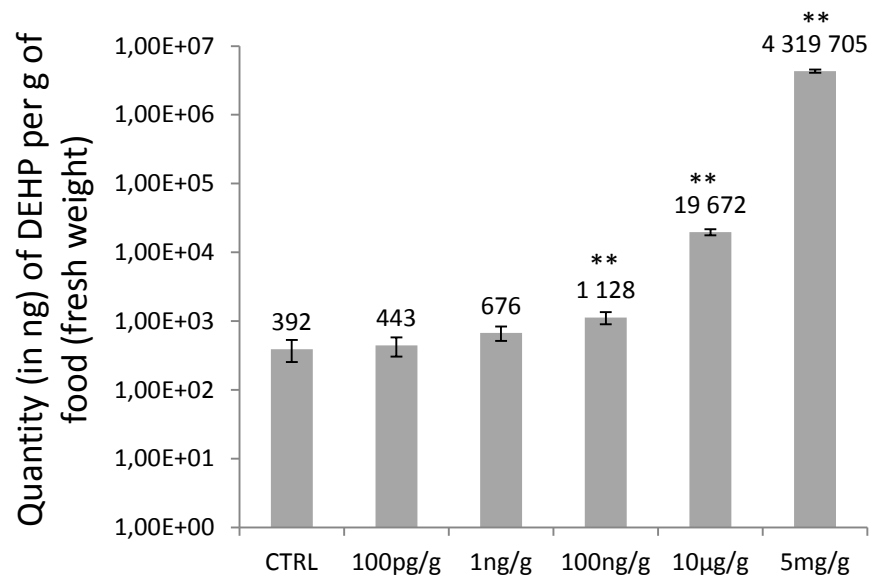
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877

Figure S1. Metabolic variations in treated and control larvae during the 5th, 6th and 7th day of the last larval stage. Hemolymph of treated and control larvae were collected, pooled and analyzed by GC-MS. Metabolite contents (nmoles. μ L⁻¹ of hemolymph) were expressed as means \pm SEM (N = 10 replicates for each experimental condition).

Figure 1

A



B

| | | | | | | | | |
|--|------------|------------|------------|------------|------------|-------------|------------|--------------------|
| Theoretical (per g of fresh food) | 100 pg | 1ng | 100 ng | 1 µg | 10µg | 500 µg | 5 mg | 40 mg |
| Measured(*) or estimated(e) by linear regression (per g of fresh food) | 443 ng (*) | 676 ng (*) | 1.1 µg (*) | 3.2 µg (e) | 19.7µg (*) | 447.2µg (e) | 4.3 mg (*) | 39.5 mg (e) |

Figure 2

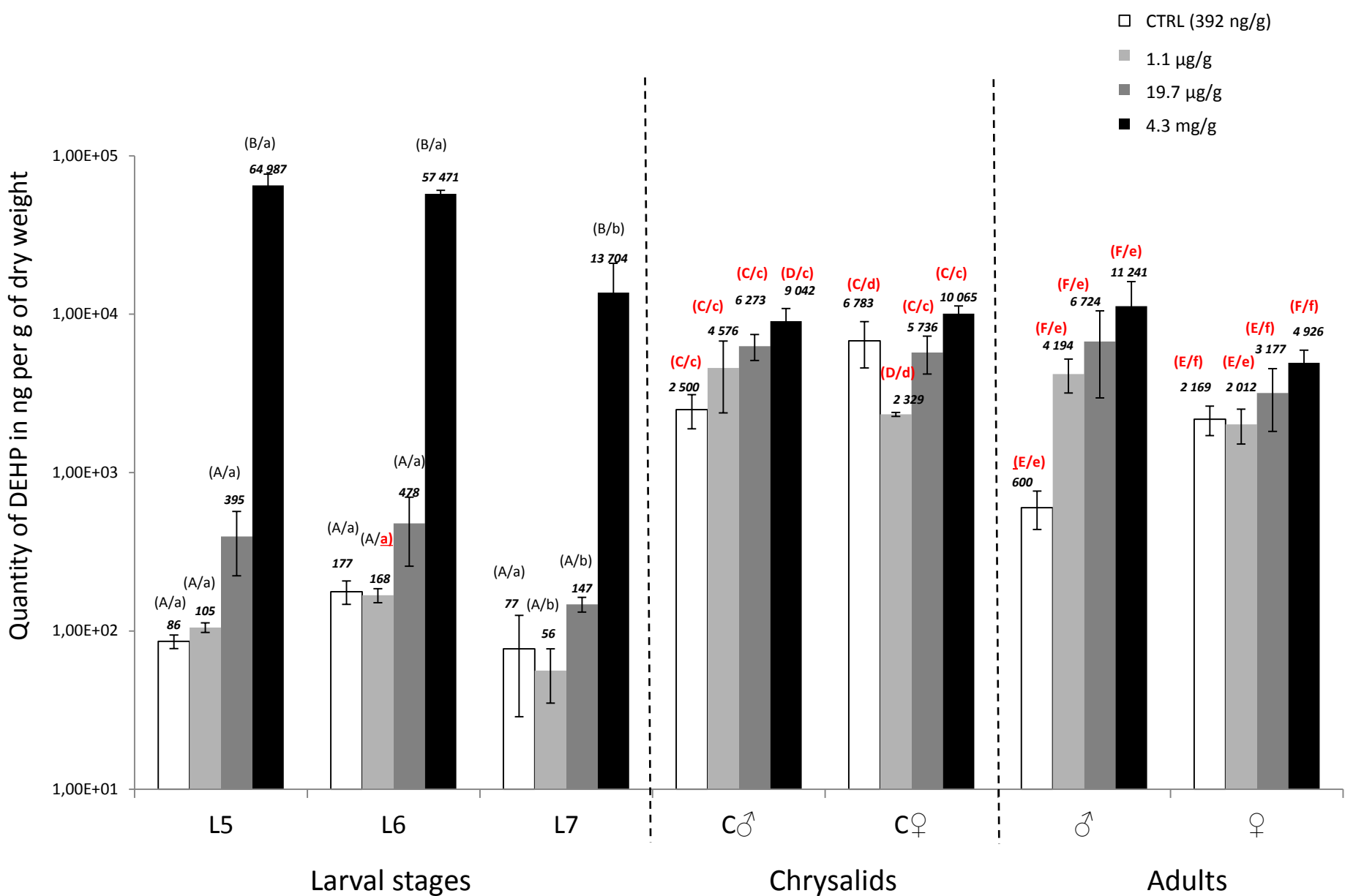


Figure 3

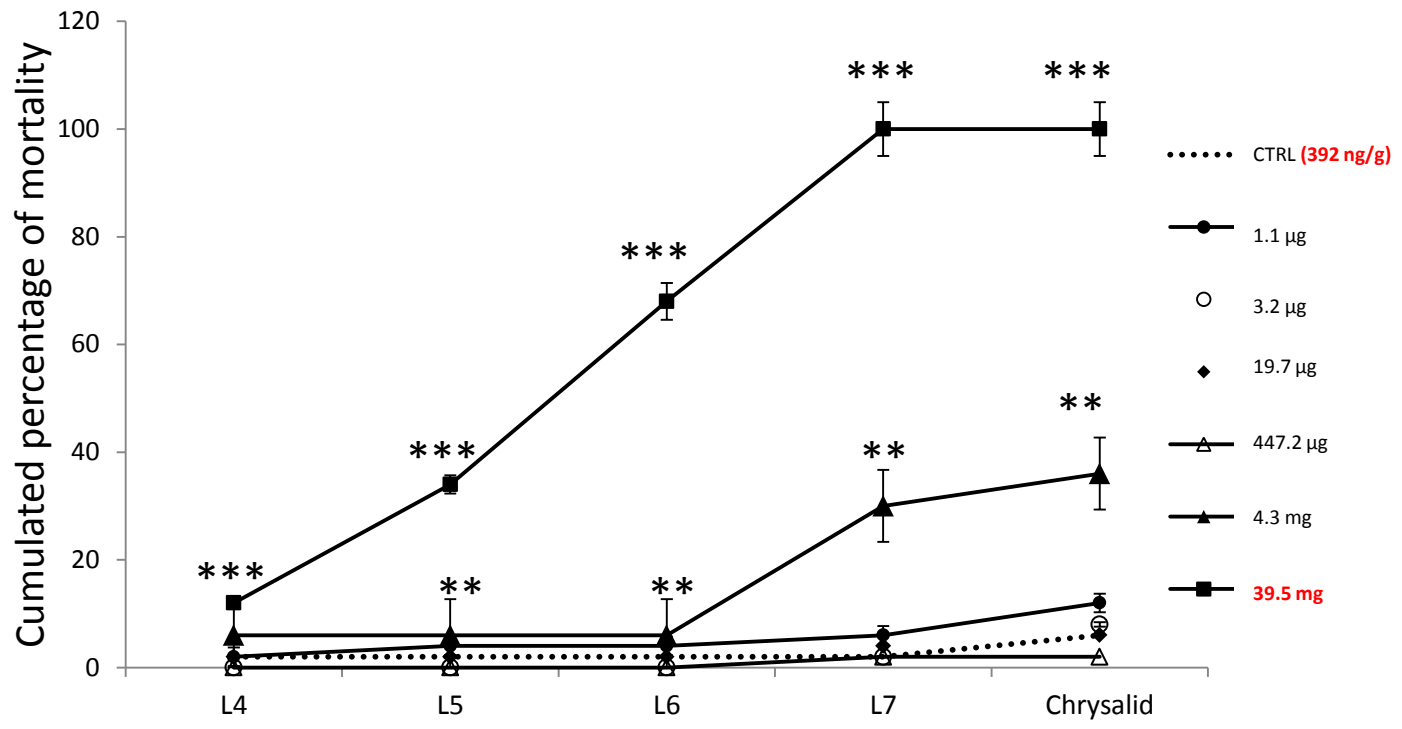
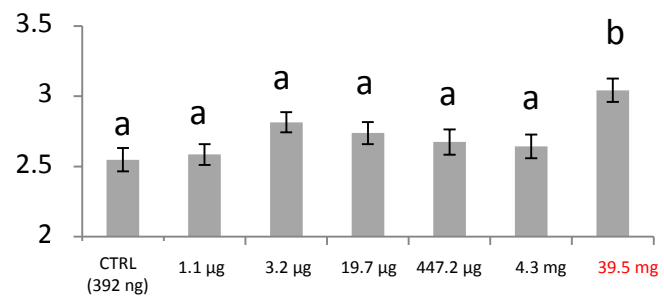


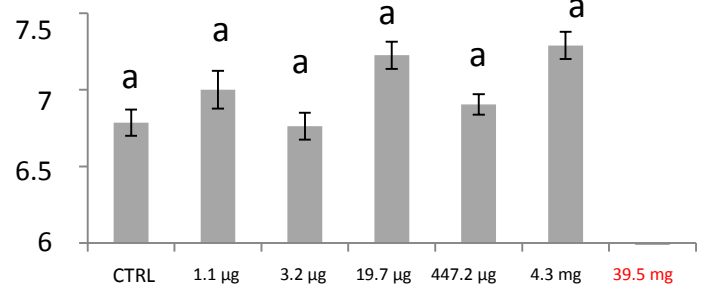
Figure 4

Duration
(in days)

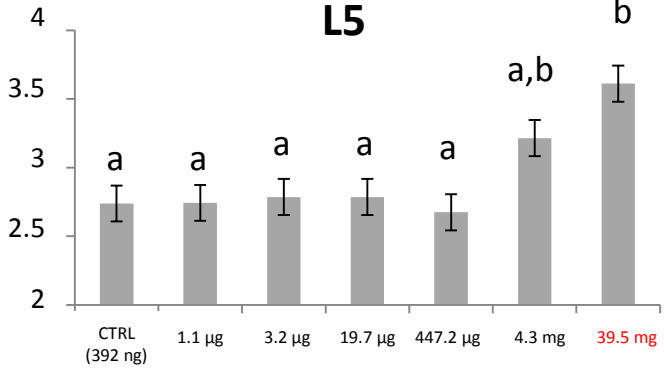
L4



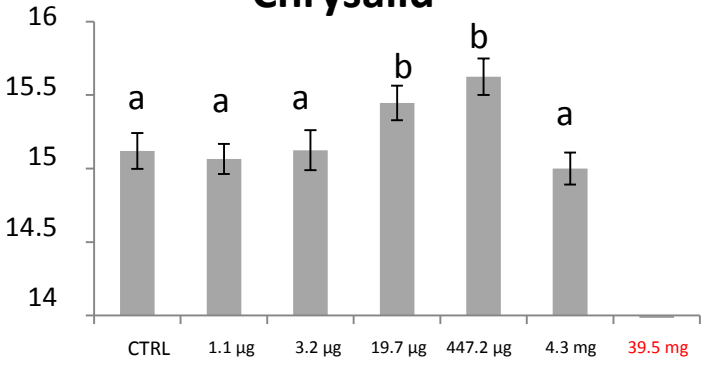
L7



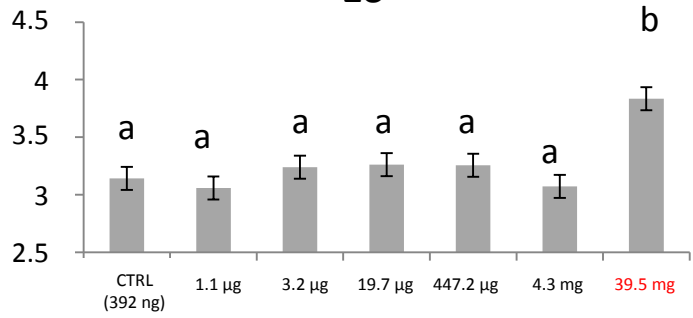
L5



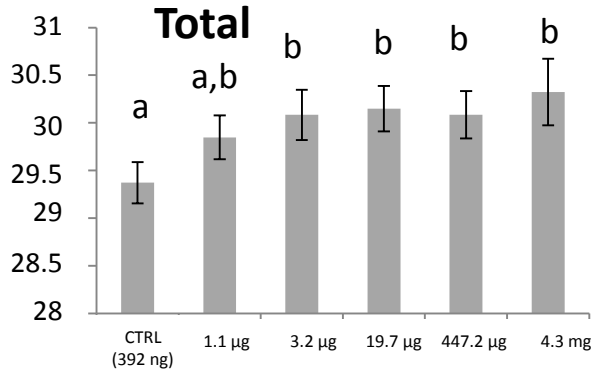
Chrysalid



L6



Total



Quantity of DEHP per gram of food

Figure 5

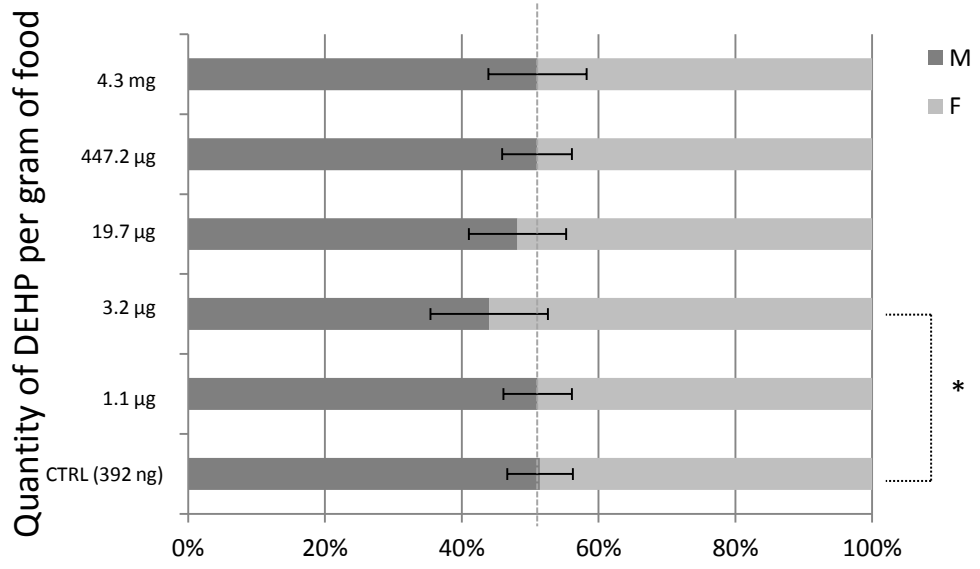


Figure 7

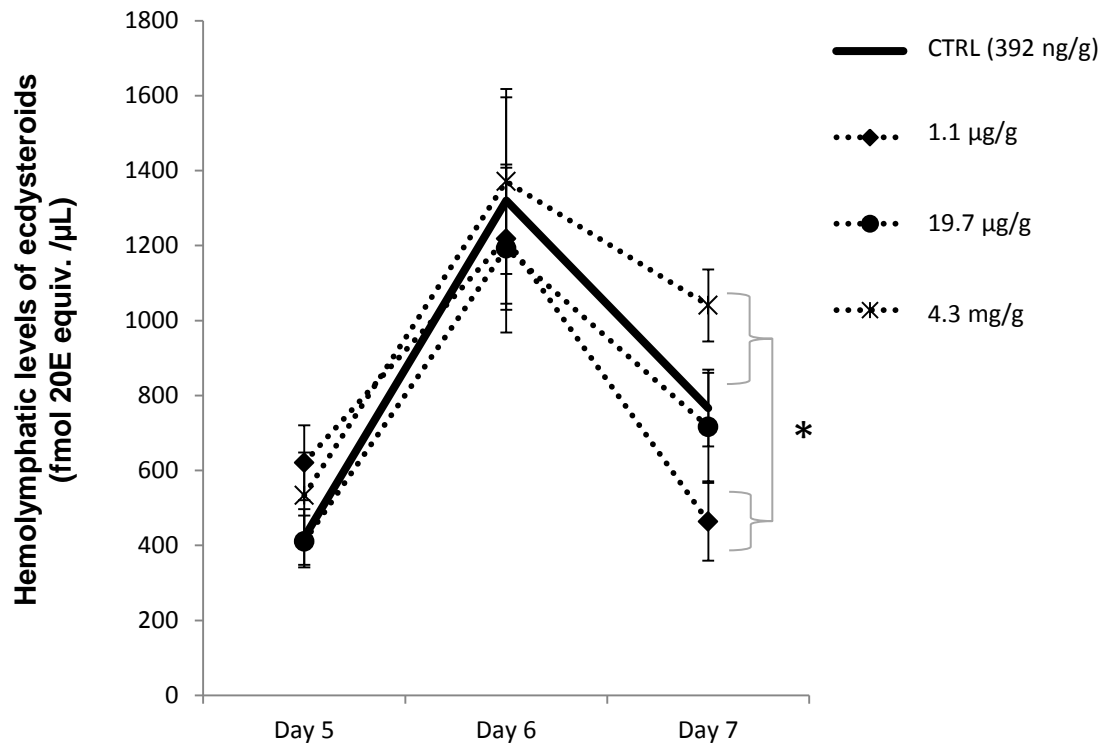


Figure 8

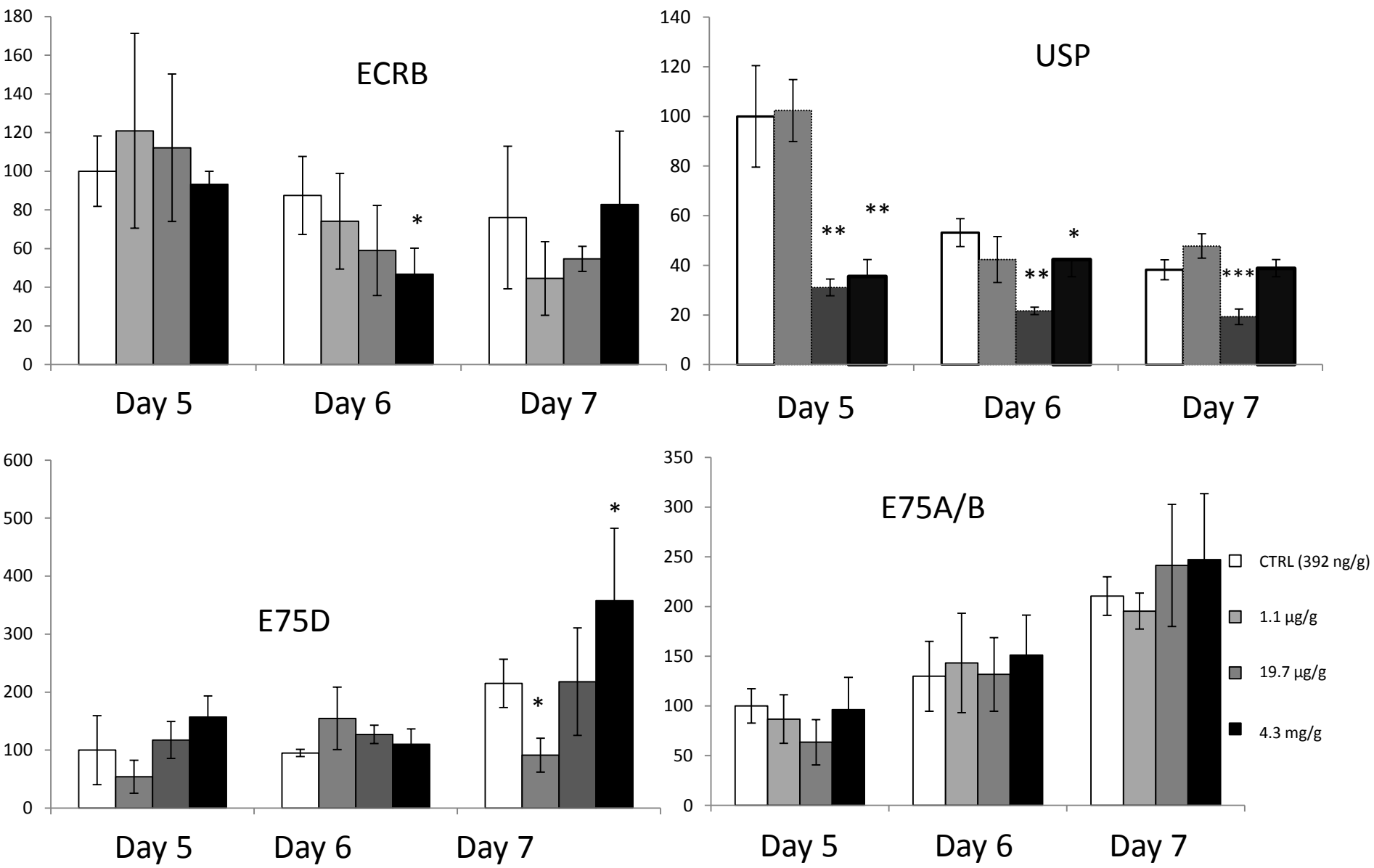


Figure 9

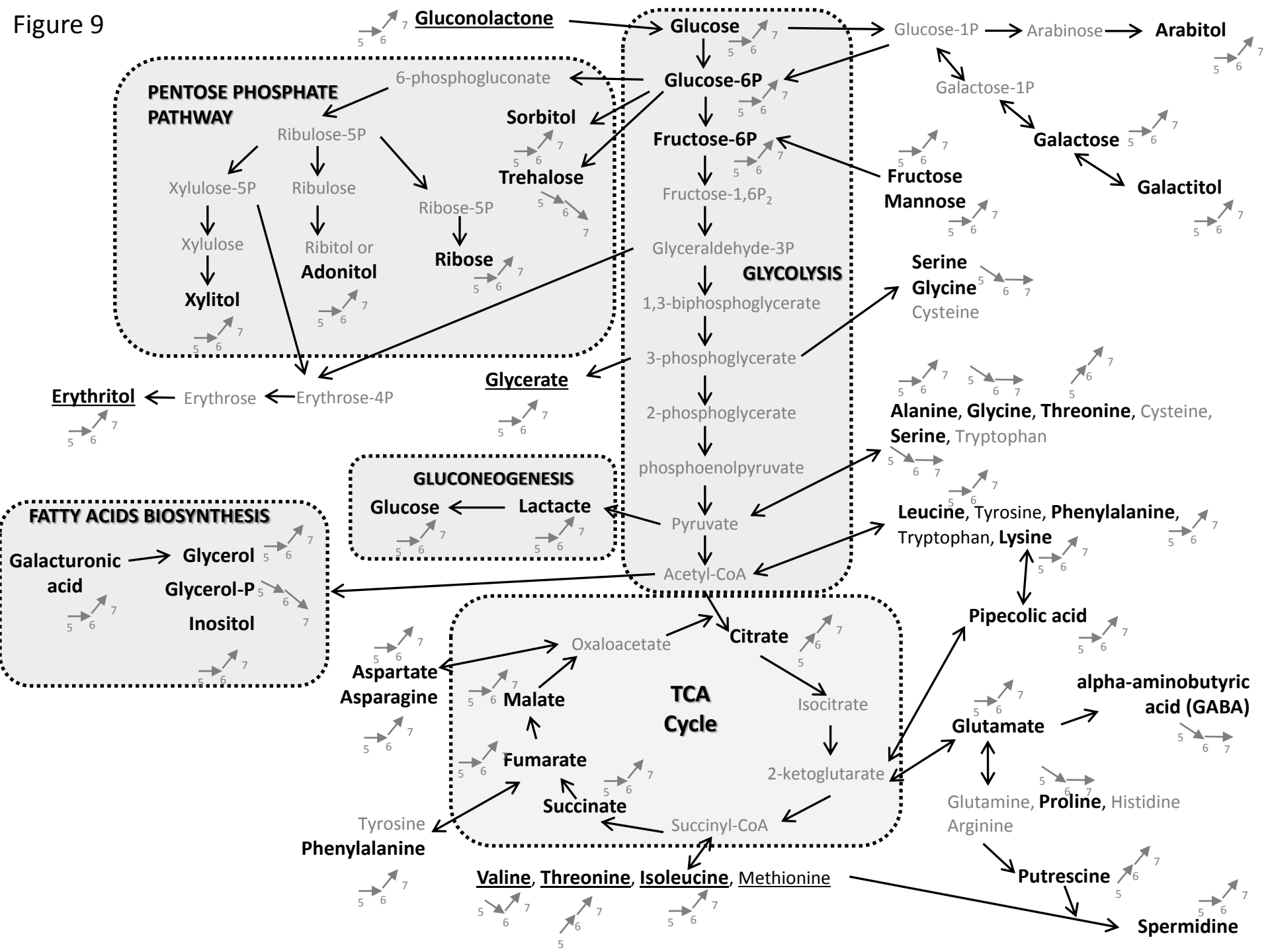


Table 1

| | | Day 5 | | | Day 6 | | | Day 7 | | |
|---------------|---------------------------|---------|----------|---------|---------|----------|---------|---------|----------|---------|
| | | 1.1µg/g | 19.7µg/g | 4.3mg/g | 1.1µg/g | 19.7µg/g | 4.3mg/g | 1.1µg/g | 19.7µg/g | 4.3mg/g |
| Adonitol | Pentose Phosphate pathway | | | | <* | | | | | |
| Alanine | Amino acid synthesis | | | | <* | <* | | | | |
| Asparagine | Amino acid synthesis | | | | <* | | | | | |
| Citric acid | TCA Cycle | | | | <* | <** | | | | |
| Fructose | Glycolysis | | | | <* | | | | | |
| Fumaric Acid | TCA Cycle | | | | <* | | | | | |
| Galacticol | Sugar alcohol synthesis | | | | <* | | | | | |
| Glyceric acid | Amino acid synthesis | | | | <* | | | | | |
| Glycerol | Fatty acids biosynthesis | | | | <* | <* | | | | |
| Lactic Acid | Gluconeogenesis | | | | | | | | | >* |
| Leucine | Amino acid synthesis | | | | <** | | | | | |
| Malic acid | TCA Cycle | | | | <* | | | | | |
| Phenylalanine | Amino acid synthesis | | | | <* | | | | | |
| Putrescine | Polyamine production | | | | <** | <** | | | | |
| Serine | Amino acid synthesis | | | | | <* | | | | |
| Sorbitol | Pentose Phosphate pathway | | | | <* | | | | | |
| Succinic acid | TCA Cycle | | | | <* | | | | | |
| Threonine | Amino acid synthesis | | | | <* | | | | | |
| Trehalose | Pentose Phosphate pathway | | | | | | | | | >* |
| Valine | Amino acid synthesis | | | | <* | <* | | | | |

Table S1

| Injector | Guard column | Column | Oven | Helium | Detector | Quantification (bold), qualification (normal), and IS (<i>italic</i>) | Reference |
|---------------|---|---|--|---------|----------|---|---------------------|
| 290°C, 1µL | Deactivated silica pre-column (1m; 0.25mm ID from Restek) | ZB-SemiVolatils (30m, 250µm film thickness from Phenomenex) | 50°C (1 min), 30°C/min to 280°C then 15°C/min to 310°C, held for 4 min | 1mL/min | EI 70eV | 149, 279 153, 171 | (Teil et al., 2013) |

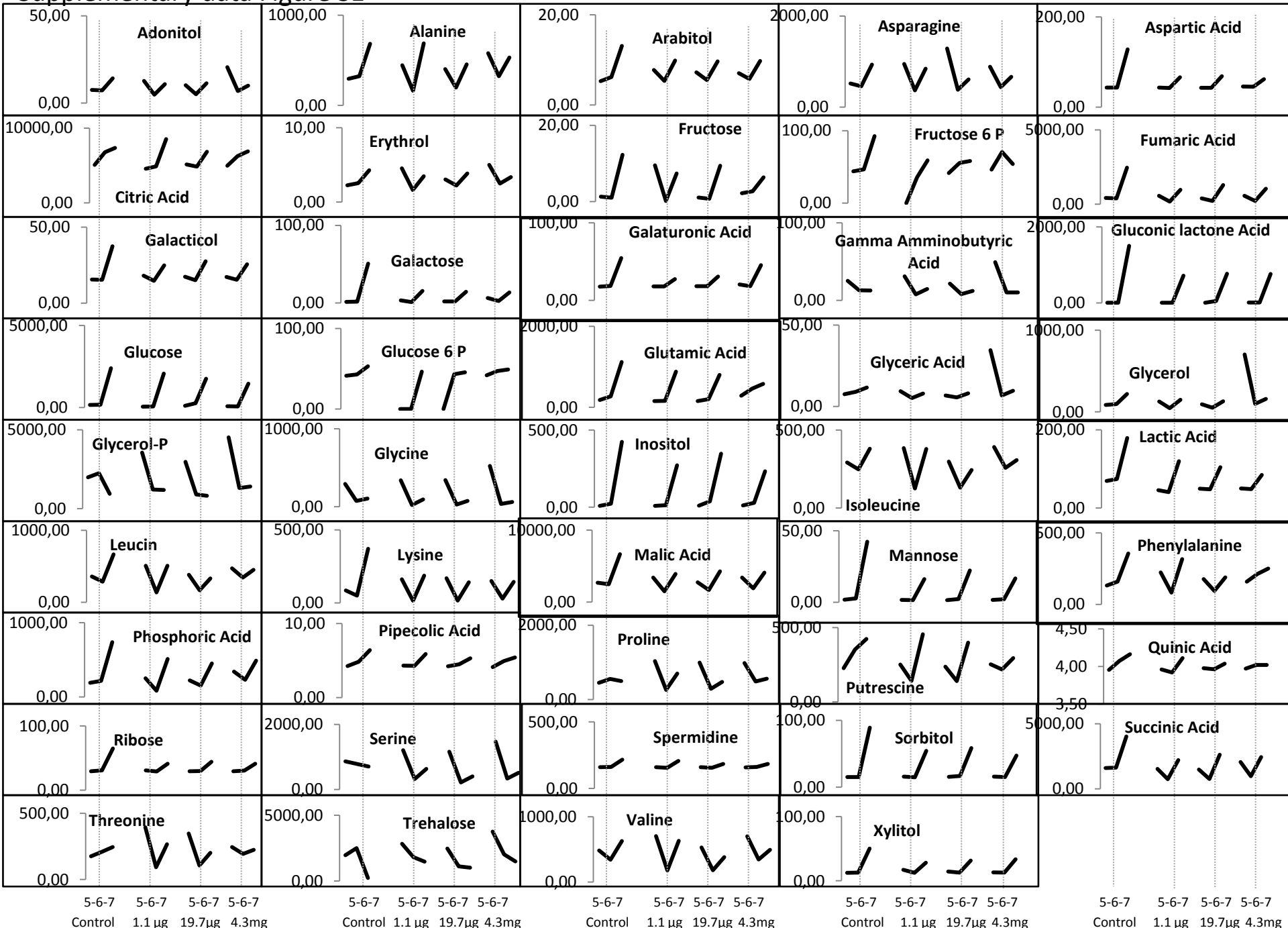
Table S2

| Primer name | Description | DNA sequence (5' – 3') |
|--------------------|-------------------------------------|-------------------------------|
| EcRB | Ecdysone receptor | F TGCAGGAAAAAGTGAAGTG |
| | | R TTCCGGGGACATTACCATAG |
| USP | Ultraspiracle | F CATGTCAGTGGCGAAGAAAG |
| | | R CCAGCGAACAGTCAACAGTC |
| E75A/B | Early response gene E75 isoform A/B | F TGGACACCCTTCCTGATCTC |
| | | R CTTCACCGGACTCACTGCTT |
| E75D | Early response gene E75 isoform D | F GTTCCGACATGGGAGAAGA |
| | | R AGGGGCATCATCTAGCTCTG |

Table S3

| Metabolites | Minimum and maximum metabolite concentrations (nmoles / μ L of hemolymph) | Metabolites | Minimum and maximum metabolite concentrations (nmoles / μ L of hemolymph) |
|----------------------------|--|-----------------|--|
| Adonitol | 0.65 - 12.84 | Lysine | 0.78 - 193.46 |
| Alanine | 14.31 - 603.90 | Malic acid | 117.43 - 1766.75 |
| Arabinose | Non detected < LQ (5 μ M) | Mannose | 0.14 - 13.14 |
| Arabitol | 0.74 - 4.31 | Melezitose | Non detected < LQ (5 μ M) |
| Asparagine | 58.36 - 725.04 | Melobiose | Non detected < LQ (5 μ M) |
| Aspartic acid | 6.89 - 37.32 | Phenylalanine | 8.03 - 153.18 |
| Cadaverine | Non detected < LQ (5 μ M) | Phosphoric acid | 9.47 - 219.82 |
| Citric acid | 467.12 - 1984.26 | Pipecolic acid | 0.65 - 1.66 |
| Citrulline | Non detected < LQ (5 μ M) | Proline | 25.48 - 784.13 |
| Dopamine | Non detected < LQ (5 μ M) | Putrescine | 13.17 - 179 |
| Erythrol | 0.16 - 3.25 | Ornithine | Non detected < LQ (5 μ M) |
| Ethanolamine | Non detected < LQ (5 μ M) | Quinic acid | 0.65 - 0.74 |
| Fructose | Non detected < LQ (5 μ M) - 22.85 | Raffinose | Non detected < LQ (5 μ M) |
| Fructose 6P | Non detected < LQ (5 μ M) - 25.86 | Ribose | 4.63 - 17.44 |
| Fumaric Acid | 12.93 - 735.59 | Saccharose | Non detected < LQ (5 μ M) |
| Galacticol | 2.38 - 10.07 | Serine | 14.56 - 566 |
| Galactose | 0.12 - 14.31 | Sorbitol | 2.40 - 28.50 |
| Galacturonic acid | Non detected < LQ (5 μ M) - 10.50 | Spermidine | Non detected < LQ (5 μ M) - 55.80 |
| γ aminobutyric acid | 0.90 - 42.13 | Spermine | Non detected < LQ (5 μ M) |
| Gentobiose | Non detected < LQ (5 μ M) | Succinic acid | 41.18 - 1194.87 |
| Gluconic lactone acid | 0.54 - 441.23 | Threonine | 9.50 - 357.98 |
| Glucose | 1.82 - 712.40 | Trehalose | 3.45 - 1209.93 |
| Glucose 6P | Non detected < LQ (5 μ M) - 11.50 | Triethanolamine | Non detected < LQ (5 μ M) |
| Glutamic acid | 15.91 - 532.64 | Tryptophane | Non detected < LQ (5 μ M) |
| Glutamine | Non detected < LQ (5 μ M) | Uric Acid | Non detected < LQ (5 μ M) |
| Glyceric acid | 0.61 - 29.30 | Valine | 12.46 - 367.06 |
| Glycerol | 3.83 - 603.45 | Xylitol | 2.04 - 15.86 |
| Glycerol P | 33.09 - 1989.48 | Xylose | Non detected < LQ (5 μ M) |
| Glycine | 1.57 - 238.97 | | |
| Inositol | 0.66 - 191.35 | | |
| Isoleucine | 8.96 - 197.27 | | |
| Lactic Acid | 2.47 - 57.25 | | |
| Leucine | 9.94 - 317.30 | | |
| Lysine | 0.78 - 193.46 | | |

Supplementary data Figure S1



Highlights

- (1) DEHP was shown to be present both in larvae and resulting stages, with higher concentrations in chrysalises and adults than in larvae.
- (2) DEHP concentrations also decreased at the end of the last larval instar, suggesting the metabolic transformation or excretion of this chemical during this time.
- (3) Only the two highest DEHP doses induced higher insect mortality, whereas low and intermediate concentrations increased larval food consumption without affecting body weight.
- (4) DEHP treatments also alter both hemolymphatic ecdysteroid titers and expression levels of ecdysteroid response genes.
- (5) DEHP can alter insect post-embryonic development and metamorphosis, by interfering with ecdysteroid pathways.