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C. Fourgous, M. Chevreuil, F. Alliot, E. Amilhat, E. Faliex, S. Paris-Palacios,
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1 **Phthalate metabolites in the European eel (*Anguilla anguilla*) from**
2 **Mediterranean coastal lagoons**

3
4 Fourgous C.¹, Chevreuil M.¹, Alliot F.¹, Amilhat E.^{2,3}, Faliex E.^{2,3}, Paris-Palacios S.⁴, Teil
5 M.J.¹, Goutte A.¹

6
7 ¹ École Pratique des Hautes Études (EPHE), SPL, UPMC Univ Paris 06, UMR 7619 METIS,
8 F-75005, 4 place Jussieu, Paris, France

9 ² Univ. Perpignan Via Domitia, Centre de Formation et de Recherche sur les
10 Environnements Méditerranéens, UMR 5110, F-66860, Perpignan, France

11 ³ CNRS, Centre de Formation et de Recherche sur les Environnements Méditerranéens, UMR
12 5110, F-66860, Perpignan, France

13 ⁴ Université de Reims Champagne Ardenne, UMR-I 02, Stress Environnementaux et
14 BIOSurveillance des milieux aquatiques (SEBIO), CNRS, Reims, France

15
16
17 Correspondence: Aurélie Goutte, aurelie.goutte@ephe.sorbonne.fr

18 Tel : +33 (0)1 44 27 63 20

19 **Abstract**

20 The levels and fate of phthalate metabolites have been poorly evaluated in fish, despite their
21 potential ecotoxicological impacts. The present study aims to characterized the levels of
22 phthalate metabolites in muscle tissue of yellow eels (*Anguilla anguilla*) from two coastal
23 Mediterranean lagoons, during three sampling periods. Nine phthalate metabolites were
24 detected in more than 70% of the samples. Slightly higher levels of phthalate metabolites
25 were detected in March and June compared to October, suggesting possible seasonal
26 variations in environmental release and/or phthalate metabolization process by eels. The large
27 sample size (N = 117) made it possible to explore correlations between phthalate metabolites'
28 levels and individual parameters, such as body length, age, body condition and hepatic histo-
29 pathologies. Body length and estimated age poorly correlated with phthalate metabolites,
30 suggesting that eels did not accumulate phthalates during growth, contrary to persistent
31 compounds. Eels presented different grades of hepatic fibrosis and lipidosis. A negative
32 correlation was found between the severity of these pathologies in the liver and the sum of
33 phthalate metabolites levels, supporting the hypothesis that eels with damaged liver are less
34 able to metabolize xenobiotics.

35

36 **Keywords**

37 Phthalate monoesters, plasticizers, by-product, fish

38

39 **Highlights**

- 40
- 41 • Phthalate metabolites were characterized in 117 European eels' muscle samples.
 - 42 • Phthalate metabolites' levels were higher in March/June than in October.
 - 43 • Body condition, length and age did not explain variations in metabolites' levels.
 - 44 • Phthalate metabolites' levels declined with increasing lesions in the liver tissue.

45 1. Introduction

46 Some synthesized chemicals are rapidly transformed through photodegradation,
47 bacterial degradation and metabolization, which may limit the bioaccumulation and
48 biomagnification of the parent compound (Mackintosh et al., 2004). Because of the intensive
49 and continuous release of these degradable pollutants into the environment, some of their
50 products of transformation may pose a significant environmental concern. Hence, a recent
51 challenging task in ecotoxicology has focused on the evaluation of the fate and impacts of
52 these biodegradable chemicals and their metabolites upon wildlife.

53 Phthalates have been extensively synthesized since the 1930s and used as plasticizers
54 mainly for polyvinyl chloride (PVC), but also as solubilizing or stabilizing agents. Their
55 worldwide production was estimated to reach 8.1 million tons per year (Crinnion, 2010).
56 Phthalate are omnipresent in all environmental compartments (Mankidy et al., 2013), and
57 induce deleterious effects, including endocrine disruption (Oehlmann et al., 2009). They are
58 easily metabolized and excreted in vertebrates, such as fish (Barron et al., 1995). However,
59 phthalate toxicokinetics and interspecific differences remain poorly described. Phthalate
60 diesters are degraded by hydrolysis in the digestive lumen, which leads to a monoester. Then,
61 hydroxylation (phase I) occurs in liver and kidney through the cytochrome P-450 system
62 (Barron, 1995). At last, oxidized monoesters are conjugated (phase II, generally by
63 glucuronidation), which enhances the solubility of the compounds and facilitates their urinary
64 excretion. Monoester can also be directly conjugated (Silva et al., 2003). Erkekoglu et al.
65 (2010) reported that the monoesters might display the same toxic potencies as the parent
66 compounds, or even greater, as for mono-ethyl-hexyl phthalate (MEHP, Zhai et al., 2014).
67 Despite the potential ecotoxicological impacts, the occurrence and levels of phthalate
68 metabolites have been poorly investigated in free-living fish (Blair et al., 2009; Valton et al.,
69 2014). Moreover, little is known about the extrinsic and intrinsic factors that drive their
70 accumulation, metabolization and excretion in wildlife.

71 The present study investigates the occurrence of phthalate metabolites in the European
72 eel (*Anguilla anguilla*), according to environmental and individual parameters. European eel
73 has been listed in 2010 on the IUCN red list as a species under critical danger of extinction,
74 after the catastrophic stock decline since the 1970s. Yellow eel is used as a sentinel species
75 for monitoring the chemical status of surface waters with respect to hazardous substances
76 because of its life cycle (benthic, predatory, long-lived and semelparous fatty species,
77 Belpaire and Goemans, 2007; Geeraerts et Belpaire, 2010). To date, the levels and effects of
78 PCBs, pesticides and trace metals have been extensively studied in European eels (Geeraerts

79 et Belpaire, 2010; Amilhat et al., 2014), while the levels of phthalate parents and metabolites
80 have been virtually neglected. Moreover, eel represents a targeted species in the important
81 economic activity of fisheries and the accumulation of hazardous substances in eels may raise
82 important public health issues. In this study, eels were collected in two Mediterranean lagoons
83 that hosts an important fishing activity (37 tons for Bages-Sigean and 10 tons for Canet-Saint
84 Nazaire in 2009, Vouvé et al. 2014) and that have to deal with increasing agricultural,
85 industrial, urban and recreational pressures. This study attempts to explain individual
86 variations of nine phthalate metabolites' levels in eels muscles (N = 117) according to
87 sampling sites and periods, as well as age, length, body condition, and histological
88 abnormalities in the liver.

89

90 **2. Materials and methods**

91

92 *2.1 Study area, species and sample collection*

93 Eels were collected from two lagoons on the south-western French Mediterranean coast of the
94 Gulf of Lions (Languedoc-Roussillon). The Canet-St Nazaire lagoon (42°39'42.28''N-
95 3°1'27.51''E) has an area of 4.9 km² and average depth is 0.4 m (maximum: 1.0 m), with a
96 proportionally extended catchment area of 260 km². Bages-Sigean lagoon (43°4'24.81''N-
97 2°59'49.98''E) covers 38 km² with an average depth of 1.5 m (maximum: 3.75 m) and a
98 catchment area of 443 km².

99 Yellow eels (juveniles of *A. anguilla*) were collected at two locations in the north
100 (BN), and in the south (BS), of the Bages-Sigean lagoon as well as in on station in the Canet-
101 St Nazaire lagoon. The collection campaigns were conducted during three periods in 2010:
102 beginning of March (N=60), June (N=60) and October (N=60), using passive fyke-nets.
103 Among the 180 sampled eels, 117 muscle samples were analyzed for levels of phthalate
104 metabolites. Eels were transported to the laboratory in containers with aerated sampling site
105 water. Eels were anesthetized by speed freezing (5 min, 4-6°C) and immediately decapitated.
106 The total length (Lt) was recorded to the nearest millimeter and the total weight (W) to the
107 nearest g. A portion (10-20 g) of fillet muscle was removed from between the pectoral fin and
108 anus then stored at -20°C until further analysis. Length varied among the three sites
109 (ANOVA: $F_{2,177} = 9.9$, $p < 0.001$), with longer eels in BN and BS (mean \pm SD: 36.1 ± 3.3 cm)
110 compared to Canet (33.9 ± 3.7 cm, Tukey post hoc tests: $p < 0.001$).

111 The condition of each eel was estimated using Fulton's condition factor $K =$
112 $100.W(g)/Lt(cm)^3$ (Ricker 1975). Body condition did not differ among the three sampling

113 sites ($F_{2,177} = 2.25$, $p = 0.11$). Body condition significantly differed among sampling periods
114 ($F_{2,177} = 28.2$, $p < 0.001$), with greater body condition in June (1.8 ± 0.2) compared to March
115 (1.5 ± 0.2 , Tukey post hoc test: $p < 0.001$) and October (1.6 ± 0.2 , Tukey post hoc test: $p <$
116 0.001). The age was estimated by analyzing the sagittal otoliths. Otoliths were removed from
117 the head of each eel and cleaned with distilled water. Small otoliths (young eels) were
118 examined in toto in camomile oil under stereomicroscope. For older eels (more than 4 years
119 old) and when the reading was not clear, the otolith was embedded in resin and ground to the
120 sagittal plane and polished until the nucleus was exposed. After using an EDTA solution,
121 otoliths were stained with toluidine blue prior to microscopic examination. The age of each
122 individual was estimated by counting the winter rings (Lecomte-Finiger 1985; ICES 2009).
123 The estimated age varied between 1 and 6 years. The eels from BS were significantly older
124 (mean \pm SD: 3.1 ± 1.0) than those from BN (2.5 ± 1.0) and Canet (2.2 ± 0.9) (Kruskal Wallis
125 test and Bonferroni post hoc test: $p < 0.001$).

126

127 *2.2 Chemicals and reagents*

128 Solvents for cleaning and extraction purposes of ultrapure quality and free from phthalate
129 residues were supplied by Merck-Chimie (Fontenay-sous-Bois, France). Oasis HLB cartridges
130 (6 mL/200 mg) were provided by Waters (Guyancourt, France). Mobile phase for high
131 performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) was
132 prepared with acetonitrile (liquid chromatography – mass spectrometry quality solvent;
133 Merck), formic acid (Carlo-Erba for analysis) and ultrapure water from a Milli-Q system.
134 Seven phthalate monoesters: mono-methyl phthalate (MMP), mono-ethyl phthalate (MEP),
135 mono-iso-butyl phthalate (MiBP), mono-nbutyl phthalate (MnBP), mono-benzyl phthalate
136 (MBzP), mono-n-octyl phthalate (MnOP), MEHP and two oxidized by-products, mono (2-
137 ethyl-5-oxohexyl) phthalate (MEOHP) and mono (2-ethyl-5-hydroxyhexyl) phthalate
138 (MEHHP) were purchased from Cambridge Isotope Laboratories, Inc. (UK). A mixture of
139 four surrogates (from Cambridge Isotope Laboratories, purity $> 95\%$), 4-
140 methylumbelliferone- $^{13}\text{C}_{12}$ (4-MU- $^{13}\text{C}_{12}$), MnBP- $^{13}\text{C}_{12}$, MEHP- $^{13}\text{C}_{12}$ and MiNP- $^{13}\text{C}_{12}$, each
141 one at $25 \text{ ng}\cdot\mu\text{L}^{-1}$ in acetonitrile (ACN) was prepared and used as $10 \mu\text{L}$ per sample. β -
142 Glucuronidase from Escherichia coli K12, $200 \text{ units}\cdot\text{mL}^{-1}$ was provided by Roche Biomedical
143 (France). Basic buffer (pH of 11) was prepared with $\text{NH}_4\text{OH}/\text{ACN}/\text{H}_2\text{O}$ (0.5:50:50, v/v/v).
144 Acid buffer (pH of 2.8) was prepared with NaH_2PO_4 (1.93 g for 100 mL) in H_3PO_4 (85
145 %)/ H_2O (1:99, v/v).

146

147 *2.3 Phthalate monoesters extraction and purification*

148 In order to avoid sample contamination by phthalates the equipment used (flask, pipet, tubes)
149 was glassware or polycarbonate tubes for storage. After washing twice with acetone and with
150 n-hexane, the glassware (except for volumetric material) was baked at 400° C in a furnace, for
151 4 h and stored with glass stoppers in aluminum foils. For micro-volumes, automated
152 analytical glass syringes (Thermo Fisher Scientific, UK) were used. Moreover laboratory
153 blanks were performed every 20 samples, following the same treatment steps than the
154 samples.

155 Prior to analysis, tissues were freeze-dried for 48 h and then grounded to obtain a fine
156 powder. Surrogates were added to the muscle samples, prior to their extraction.
157 Concentrations of seven monoesters (MMP, MEP, MiBP, MnBP, MBzP, MnOP and MEHP)
158 and two phase I metabolites of DEHP (MEOHP and MEHHP) were determined in eel
159 muscles (N = 117).

160 First, 500 mg of freeze-dried muscle tissue were spiked with surrogates in a 25 mL
161 polycarbonate tube and crushed with an Ultra-Turrax in 4 mL of ammonium acetate (1 M).
162 Then, a deconjugation step was used (90 min, 37° C) with β -glucuronidase from *E. coli* K 12
163 (25 μ L) free from any detectable esterase activity upon phthalate diesters (Blount et al. 2000).
164 The glucuronide conjugate of 4-MU, the 4-methylumbelliferyl- β -D-glucuronide, was used
165 (140 mg by incubation) for controlling the deconjugation reaction. Thus, for each parent
166 phthalate, total metabolites were quantified including deconjugated compounds.

167 After deconjugation and centrifugation (4 500 RPM, 10 min), the supernatants were
168 collected into a 15 mL glass tube and 3 mL of ammonium acetate (1 M) were added to the
169 deconjugated sample and homogenized with an Ultra-Turrax, centrifuged and the supernatants
170 collected (step repeated with 2 mL of ammonium acetate (1 M)). The combined supernatants
171 were extracted twice on SPE (solid phase extraction) cartridges (Oasis HLB 6 mL/200 mg)
172 for purification. Prior to the first elution, the cartridge were conditioned with 3 mL of ACN
173 and 6 mL of basic buffer. After loading, the samples were cleaned-up (twice) with 2 mL of
174 ammonium acetate (1 M) and eluted with 6 mL of basic buffer, and collected in a 22 mL glass
175 tube with 10 mL of acid buffer. The acidified sample was then purified through a second
176 conditioned SPE cartridge (1 mL of ACN then 1 mL of H₂O and 2 mL of acid buffer) and
177 washed (twice) with 2 mL of acid buffer then 2 mL of H₂O. The analytes were eluted with 10
178 mL of ACN then, 10 mL of ethyl acetate. The purified eluate was evaporated under nitrogen
179 stream in a thermostatic bath (65° C), until dry. The residue was resuspended in 0.5 mL of
180 ACN, filtered under centrifugation (6 000 RPM, 5 min) and transferred in vial. This procedure

181 was repeated three times. Finally, the filtrated samples were combined and concentrated under
182 nitrogen stream (65° C) at 500 µL before analysis. Concentrations (ng.g⁻¹ dry weight, *dw*) are
183 given for individual phthalate metabolites as well as the sum of all nine metabolites (MPA).
184 Water content in eel muscles was 40.2 ± 6.1 %.

185

186 *2.4 LC-MS/MS analysis*

187 Phthalate monoesters were quantified by liquid chromatography (1200 series - Agilent
188 Technologies, Massy, France) – tandem mass spectrometry (6410-triple quadrupole MS, in
189 negative ion mode for the electrospray source - Agilent Technologies (LC- MS/MS),
190 equipped with a silice upti-prep strategy column (100 A, C18-2, 2x100 mm, 2.2 µm –
191 Interchim) heated at 40°C. The source was in ESI negative mode (N2: 350°C; gas flow: 660
192 L/h; capillary: 4000 V) and the injected sample volumes were 10 µL. The LC gradient flow,
193 the MRM parameters for LC-MS/MS and limits of quantification (LOQs) are indicated in the
194 Supplementary Material (Table SM.1, 2, 3 and 5).

195

196 *2.5 Method validation*

197 The analytical method performance was determined by evaluating the linearity, recovery
198 rates, instrument detection limits (IDLs), limits of detection (LODs) and limits of
199 quantification (LOQs). Performance in terms of linearity and dynamic range was checked by
200 injecting reference standard solutions in duplicate. The range tested was 0.3-10 ng on column
201 for phthalate metabolites and 0.002-2 ng for 4-MU.

202 Recovery experiments were performed on eel muscle samples (N = 3) spiked at high
203 concentration levels, i.e. at least 10 times higher than the mean concentrations of each
204 endogenous phthalate metabolite in eel muscle samples (Table SM.4, Supplementary
205 Material).

206 IDLs were determined using the less concentrated surrogate as a signal/noise ratio of 3
207 peak to peak.

208 Eight procedural blanks that underwent all the sample extraction and purification, were
209 analyzed every 20 samples. All samples were blank-corrected by subtracting the mean
210 phthalate metabolite concentration in the batch blanks from each sample, prior to recovery
211 correction. The LOD was calculated as the mean value plus three standard deviations of the
212 analyte in blank samples, and LOQ was calculated as the mean value plus ten standard
213 deviations of the analyte in blank samples.

214

215 *2.6 Histological approach*

216 Small pieces of liver from 62 eels were fixed with Bouin Hollande's solution, dehydrated with
217 graded series of ethanol, placed in butanol-1 for at least 24 h and then embedded in paraffin
218 wax. Liver sections (6 µm) were stained with nuclear fast red (NR) and picro-indigo-carmin
219 (PIC) (Gabe 1968). Microphotographies were taken with a light microscope (Laborlux S –
220 Leitz wetzlar Germany) equipped with a digital camera (Imagingsource) and analysis were
221 realized with the Archimed software (Version 5.6.0.). Three different liver areas (25 mm²)
222 randomly selected on different sections were analyzed for each eel. Scoring notes for 5
223 hepatic parameters: lysis-necrosis, lipidosis, fibrosis, melano-macrophage (MMC) aggregates
224 and nuclear alterations, were established and an individual global score was calculated as the
225 mean value of the different scores (Jaffal et al., 2015). The normal histological state of the eel
226 liver (score 0) corresponding to healthy uncontaminated yellow eels was established
227 according to studies realized 30 to 20 years before, when it was possible to collect eels that
228 were not exposed to phthalates (Barni et al., 1985; Affandi and Biagianti, 1987; Lemaire-
229 Gony and Lemaire, 1992). The higher scores of histopathological alterations were established
230 taking into account the grade of alterations reported in literature for eel but also for different
231 fish species (Affandi and Biagianti, 1987; Braunbeck et al., 1990; Costa et al., 2011; Jaffal et
232 al., 2011). The global histopathological score (on 10 grade) was calculated with the formula:
233
$$\text{Global score} = [\text{score}_{\text{lysis-necrosis}} + \text{score}_{\text{MMC}} + \text{score}_{\text{fibrosis}} + \text{score}_{\text{nucleus}} + \text{score}_{\text{lipid}}] / 5.$$

234 Lysis-necrosis score was established as follow: 0 = less than 10 %, 2.5 = 10 to 25 %; 5 = 25 to
235 50 %; 7.5 = 50 to 75 %, 10 = more than 75 % of death hepatocytes in the parenchyma. To
236 establish the nuclear alterations score, the quantity of altered nucleus among 40 randomly
237 observed was converted in a score on 10 points. The lipid score was established on the size
238 and the amount of the lipid droplets observed in hepatocytes: 0 = no lipid droplet; 2.5 = few
239 little lipid droplets; 5 = lipid droplets present in 25 to 50% of the hepatocyte; 7.5 = more than
240 50% of the hepatocytes presented large lipid droplets and have a double sized; 10 = all
241 hepatocytes were filled with huge lipid droplets and have at least a double size. The fibrosis
242 score was determined by observation of the collagen deposits localization in liver : 0= only
243 big blood vessel (portal section) had collagen deposits, 2.5 = some blood vessel and bile duct
244 presented collagen deposit, 5= the majority of the blood vessel and bile duct presented
245 collagen deposit, 7.5= abnormal large collagen deposits were observable around sinusoids,
246 veins, small bile ducts and some hepatocytes, 10= collagen deposit present throughout all the
247 parenchyma, particularly between hepatocytes. Melano macrophage aggregate (MMA) were

248 counted and the score was the quantity of MMA per area with a maximum of 10 (score10 =
249 10 or more MMC observed on each of the three areas).

250

251 *2.7 Statistical analyses*

252 All statistical tests were performed using R software version 3.2.2 (R Development Core
253 Team 2015). Data were log-transformed to make the data fit the normal distribution.
254 Significant differences among groups (i.e. sampling periods or sampling sites) were evaluated
255 by ANOVA (or Kruskal-Wallis tests for non-parametric data), followed by post hoc Tukey
256 test (or Bonferroni tests for non-parametric data) if ANOVA indicated a significant difference
257 between groups. Correlations between phthalate metabolites and individual parameters (age,
258 body condition, body length, score of histological abnormalities in liver) were expressed by
259 Pearson (or Spearman for non-parametric data) correlation coefficient.

260

261 **3. Results**

262

263 *3.1. Method performance*

264 The column separation obtained for the LC-MS/MS method were consistent with accurate
265 quantification. The Multiple Reaction Monitoring mode (MRM) separates masses in two
266 stages, making the instrument significantly more selective and sensitive than a single
267 quadrupole system. As a result, compounds can be easily identified and quantified in the
268 presence of complex matrices using MRM. Quantification of phthalate metabolites was
269 carried out by calculating the response factor for each compound relative to the corresponding
270 surrogate and concentrations were obtained using a linear regression analysis of the peak area
271 versus the concentration ratio.

272 The results for linearity were satisfactory with an R^2 ranging from 0.997 to 0.999
273 (Table SM.3, Supplementary Material). Recoveries for the overall extraction procedure
274 displayed satisfactory results (74% - 102%) for MiBP, MEHHP, MnBP, MEOHP, average
275 score for MEHP and MBzP (46% - 61%) and poor results (12% - 32 %) for MMP, MEP,
276 MnOP (Table SM.4, Supplementary Material). Low recovery rates for these phthalate
277 metabolites could be due to the long lasting protocol, and especially the deconjugation step.
278 IDLs ranged from 0.3 to 6 pg (Table SM.2, Supplementary Material). The nine phthalate
279 metabolites were detected in the 8 blanks (Table SM.5, Supplementary Material) and were
280 consistent with compound determinations. The LODs and LOQs were compatible with fish

281 concentrations and allowed quantification of all compounds (Table SM.5, Supplementary
282 Material).

283

284 *3.2. Phthalate metabolites in eel muscles*

285 All the phthalate metabolites were detected in more than 70% of the muscle samples
286 analyzed. Large inter-individual variations were detected (Table 1, Fig. 1). The most abundant
287 phthalate metabolites were MEHP, followed by MiBP and MnBP, whereas the less abundant
288 was MBzP (Table 1, Fig. 1). Positive correlations were detected between MiBP and MnBP
289 (DBP metabolites), between MEHP, MEHHP and MEOHP (DEHP metabolites), and between
290 MEOHP and MnOP (Table 2). A negative correlation was detected between MEHHP and
291 MnBP (Table 2).

292

293 *3.3. Sampling sites and periods*

294 The levels of each phthalate metabolite and of the sum of all nine metabolites in eel muscle
295 did not significantly differ among the sites Canet, South and North of Bages-Sigean (Table 3).
296 Concerning the sampling periods, levels of MEP, MEHHP and MEOHP were higher in March
297 and June than in October (Table 3, Fig. 2), whereas the other phthalate metabolites did not
298 show significant differences among the three periods (Table 3).

299

300 *3.4. Age, length, body condition*

301 Muscular levels of phthalate metabolites in eels were not correlated to age, body length and
302 body condition (Table 4), except a negative correlation between MnBP levels and age,
303 between MnBP levels and length and a positive correlation between MEOHP levels and
304 length.

305

306 *3.5. Histological abnormalities*

307 Histological abnormalities were detected in the liver of eels collected at the three stations. The
308 main liver alterations were hepatocytic lysis or necrosis, nuclear abnormalities, lipid
309 accumulation, fibrosis and presence of melano-macrophage aggregates. The score of
310 histopathological perturbations in eels revealed clear liver pathologies in most of the
311 individuals. The higher grade of hepatic alterations were related to important accumulation of
312 lipid forming large lipid droplets in enlarged hepatocyte and paralleled with nuclear alteration,
313 fibrosis and necrosis and lysis which characterize the lipoid degeneration (also called fatty

314 liver) (Fig.3). Muscular levels of MPA, MEP, MiBP and MnOP significantly decreased with
315 increasing number of hepatic abnormalities (Table 4, Fig. 4).

316

317 **4. Discussion**

318

319 *4.1. Muscular levels of phthalate metabolites*

320 The detection of phthalate metabolites in eel muscle suggested that eels were directly exposed
321 to phthalate metabolites from the water or the sediment, since phthalate parents can be
322 transformed by microorganisms, or that eels were exposed to phthalate parents and were able
323 to incorporate and metabolize them. MEHP, MiBP and MnBP were the major phthalate
324 metabolites. Results obtained in this study and using a new method are consistent with
325 previous studies that reported similar muscle contents of phthalate metabolites in fish
326 collected in an urbanized marine inlet, in Vancouver: the juvenile Shiner Perch *Cymatogaster*
327 *aggregata* (geometric mean ranged from 0.54 ng.g⁻¹ ww. for MBZP to 82 ng.g⁻¹ ww. for
328 MnBP, McConnell, 2007) and the white spotted greenling *Hexagrammos stelleri* (0.24-1.1
329 ng.g⁻¹ ww for MEHP, 6.63-60.9 ng.g⁻¹ ww for MnBP, Blair et al., 2009), as well as in marine
330 fish from the Yangtze River Delta sea area (China, 1.2 to 20.4 ng.g⁻¹ ww, Gu et al., 2014). It
331 should be noted that much higher levels of phthalates metabolites were found in liver samples
332 (mean ranged from 7.1 ± 2.6 (MEOHP) to 1607 ± 2706 (MiBP) ng.g⁻¹ dw) of roaches fished
333 in the Seine river, France (Valton et al., 2014), which is coherent with the metabolization of
334 phthalates in liver.

335

336

337 *4.2. Seasonal and spatial variations*

338 Lagoons are known to be characterized by great spatial and seasonal variabilities in
339 environmental conditions (e.g. temperature, Viaroli et al., 2007). In the present study no
340 significant differences of phthalate metabolite levels were observed among the three sites.
341 This lack of spatial difference could be due to the geographical proximity of Canet and Bages
342 Sigean lagoons and the absence of direct discharge of urban wastewater into these lagoons.
343 The levels of PCBs and PBDEs were also relatively low in the sediment and eels muscles
344 from these two lagoons (Labadie et al., 2010; Vouvé et al., 2014). Analyses of phthalate
345 concentrations in water and sediments should be necessary to confirm this lack of spatial
346 variation.

347 A temporal variation was detected, especially for MEP, MEOHP and MEHHP, whose
348 levels were higher in March/June than in October 2010. This could be due to a seasonal
349 change in exposure to phthalate, in abiotic factors (e.g. temperature, precipitation, salinity,
350 oxygen rate) and thus in the hepatic enzymatic activities involved in detoxification processes
351 in fish (Förlin et al.,1995). Moreover, March/June corresponds to the intense recreational
352 activity, and potentially to an increasing release of plasticizers into the lagoons. Additional
353 data are needed, however, to confirm this seasonal trend

354

355 4.3. Age, length, body condition

356 The large sample size of eel muscles (N = 117) made it possible to explore correlations
357 between phthalate metabolites' levels and individual parameters. Age was not correlated to
358 phthalate metabolites' levels, except for MnBP, whose levels decreased with age. Similarly
359 body length was not correlated to muscular levels of phthalate metabolites, except for MnBP
360 whose levels decreased with length and for MEOHP whose levels increase with length. These
361 results suggest that eels did not accumulate phthalates during growth, contrary to persistent
362 compounds. For instance, levels of polychlorobiphenyls (PCBs) and polybrominated
363 diphenyl-ethers (PBDEs) increased with length and age from glass eels to silver eels (Tapie et
364 al., 2011). Similarly, PCBs, hexachlorocyclohexanes (HCHs), hexachlorobenzene (HCB),
365 dichlorodiphenyltrichloroethane (DDT) and dichlorodiphenyldichloroethylene (DDE)
366 accumulated with age in the muscle of brown trout (*Salmo trutta*, Vives et al., 2005). It should
367 also be noted that range of age and length were limited in our data set, which may have
368 restricted patterns of accumulation or dilution with growth. At last, eels could have been
369 exposed to phthalate intermittently throughout their lives.

370 No correlation were found between muscular levels of phthalate metabolites and body
371 condition, suggesting that metabolization efficiency is not linked to the individual body
372 condition.

373

374 4.4 Histological abnormalities

375 An original and innovative objective of this study was to explore whether individuals
376 with hepatic lesions had lower metabolites' levels, because of a less efficiency of their liver to
377 metabolize phthalate parents. The prevalent histopathological characteristics identified in eel
378 from Mediterranean lagoons were hepatocellular necrosis and parenchymal lysis, cellular
379 hyperplasia associated to lipid vacuolization and fibrosis who are interpreted as nonspecific
380 responses to stress as they have been described in fish exposed to a wide spectrum of

381 pollutants (Hinton et al., 1992). The lipoid degeneration (fatty liver) is one of the major
382 changes observed here. In yellow eel, this change is symptomatic of a metabolic dysfunction
383 since their liver does not usually store lipids. Lipid droplets may sequester fat-soluble
384 pollutants and may reduce the metabolic capacity of that tissue. Moderate lipidosis is
385 commonly regarded as an unspecific alteration with multiple potential causes and lipoid
386 degeneration indicates a severe alteration of fish health (Costa et al., 2011). Lipoid
387 degeneration is linked to an high rate of hepatic alteration and fatty liver probably take place
388 with impairment of their function (Vandemiaie et al., 2001).

389 Muscular levels of the sum of phthalate metabolites were negatively correlated with
390 the score of histological abnormalities in the liver. This negative correlation supports the
391 hypothesis that individuals with numerous hepatic lesions are less efficient to metabolize
392 xenobiotics. Such hepatic alteration of the metabolic function would imply an accumulation
393 of phthalate parents.

394

395 **Conclusion**

396 Nine phthalate metabolites were characterized in muscle tissue of 117 European eels. No
397 difference was found between the two lagoons, and a weak temporal variation was detected
398 with higher contamination in March/June than in October. Body condition, length and age did
399 not explain individual variations in muscular levels of phthalate metabolites suggesting that
400 eels did not accumulate phthalates during growth, contrary to persistent compounds..
401 Individuals with numerous histological abnormalities in liver bore lower levels of phthalates
402 metabolites, suggesting that they were less efficient to metabolize xenobiotics. Further
403 ecotoxicological research should be conducted to improve understanding of the links between
404 fish physiology and ecology and the metabolization of phthalates.

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409

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513 zebrafish larvae after exposure to mono-(2-ethylhexyl) phthalate (MEHP). *PloS one*, 9(3)
514 e92465..

516 Table 1. Mean \pm standard deviation (SD) of phthalate metabolites in eel muscles ($\text{ng}\cdot\text{g}^{-1}$ dw,
 517 N = 117). % represents the percentage for each metabolites with respect to the sum of
 518 phthalate metabolites (MPA).

519

	MPA	MMP	MEP	MiBP	MnBP	MBzP	MnOP	MEHP	MEHHP	MEOHP
Mean	913	5.7	33	206	174	2.0	82	282	94	34
SD	885	6.0	108	280	333	4.1	162	403	206	94
%		0.6%	3.6%	23%	19%	0.2%	9%	31%	10%	3.7%

520

521

522 Table 2. Pearson or Spearman correlation matrix between phthalate metabolites levels in eel
 523 muscles (N = 117, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

524

	MEP	MiBP	MnBP	MBzP	MEHP	MEHHP	MEOHP	MnOP
MMP	0.1	0.03	- 0.15	0.05	0.2 **	0.2 **	0.1	0.11
MEP		0.3 **	0.1	0.15	0.1	0.2	0.2	0.08
MiBP			0.7 ***	0.4 ***	- 0.02	0.1	0.15	0.01
MnBP				0.2 **	- 0.02	- 0.2 *	0.2	0.2*
MBzP					0.2 *	0.2	0.2 *	0.02
MEHP						0.5 ***	0.4 ***	0.2 *
MEHHP							0.5 ***	- 0.009
MEOHP								0.5 ***

525

526

527 Table 3. Spatial and temporal differences in the levels of phthalate metabolites in eel muscles,
 528 using ANOVA or Kruskal Wallis (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). When the
 529 difference was statistically significant within sites or periods, a Tuckey or Bonferroni post hoc
 530 test was realised.

531

	MPA	MMP	MEP	MiBP	MnBP	MBzP	MEHP	MEHHP	MEOHP	MnOP
Periods	$F_{2, 114} = 3.3 *$	$X^2 = 4.4 **$	$X^2 = 18.1 ***$	$F_{2, 114} = 1.4$	$X^2 = 2.2$	$X^2 = 2.7$	$X^2 = 5.1$	$X^2 = 7.6 *$	$F_{2, 114} = 8.7 ***$	$X^2 = 1.6$
Post-hoc			Oct<Jun ** Oct<Mar ***					Oct<Jun *	Mar<Jun * Oct<Jun ***	
Sites	$F_{2, 114} = 0.3$	$X^2 = 0.9$	$X^2 = 1.3$	$F_{2, 114} = 1.8$	$X^2 = 5.6$	$X^2 = 2$	$X^2 = 0.6$	$X^2 = 0.08$	$F_{2, 114} = 0.3$	$X^2 = 1$

532

533 Table 4: Pearson or Spearman correlation matrix between the levels of phthalate metabolites
 534 in eel muscles and individual parameters (age, length, body condition and the score of
 535 histological abnormalities in the liver (histo), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

536

	MPA	MMP	MEP	MiBP	MnBP	MBzP	MEHP	MEHHP	MEOHP	MnOP
Age	- 0.02	- 0.1	- 0.08	- 0.05	- 0.2 *	- 0.07	0.08	0.15	0.2	0.05
L	- 0.07	0.04	- 0.03	- 0.02	- 0.25 **	- 0.2	0.01	0.1	0.2 *	- 0.02
K	- 0.08	- 0.09	- 0.1	- 0.09	- 0.1	- 0.1	- 0.03	0.05	0.1	0.07
Histo	- 0.4 **	- 0.1	- 0.3 **	- 0.3 *	- 0.2	- 0.2	- 0.07	- 0.05	- 0.01	- 0.3 *

537

538

539 Figure legend

540

541 Figure 1: Distribution of phthalate metabolites content (log-transformed) in muscle ($\text{ng}\cdot\text{g}^{-1}$
542 dw) in 117 eels specimens.

543

544 Figure 2. Seasonal distribution of phthalate metabolites content (log-transformed) in 117
545 muscle samples ($\text{ng}\cdot\text{g}^{-1} dw$).

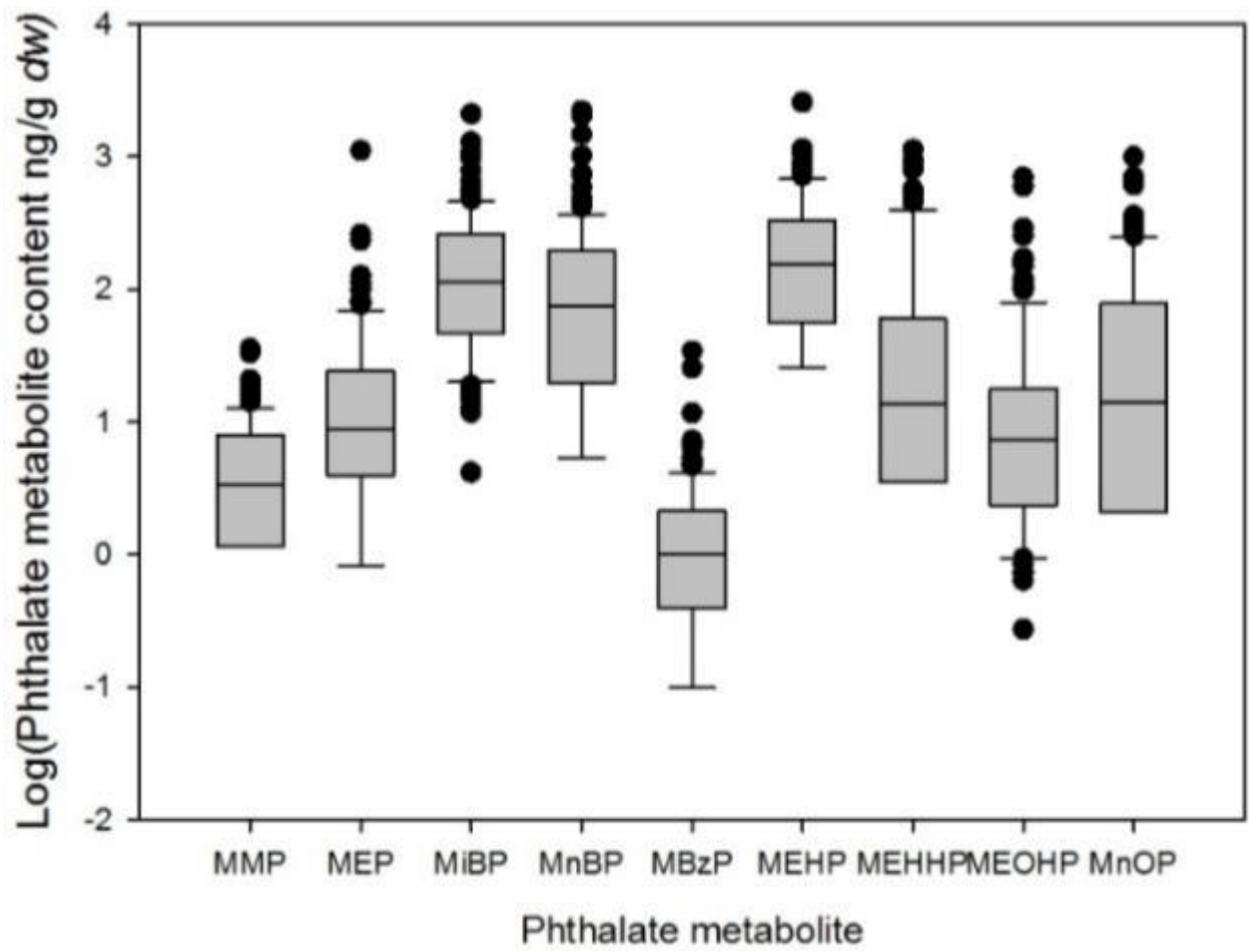
546

547 Figure 3. Micrographies of eel liver showing contrasted grade of hepatic alterations : [A] low
548 perturbations in liver of an eel from BN in March (score 1/10); [B] moderate grade of
549 alterations in the liver of an eel from BN in June with a beginning of lipidosis (score 3/10);
550 [C] steatosis in liver of eel from BS in October (score 5/10) and [D] high grade of alterations
551 in liver of an eel from C in October undergoing lipoid degeneration with lipidosis, fibrosis
552 and frequent necrosis and lysis (score 7/10). Bar = 20 μm , AN : altered nucleus, FV: fibrotic
553 vessel, MMA: melanomacrophage aggregate, nN : normal nucleus, N: necrosis, Ly: lysis, li:
554 lipidic droplet, V: vessel.

555

556 Figure 4. Correlations between the sum of all the phthalate metabolites (MPA) content in
557 muscle (log-transformed, $\text{ng}\cdot\text{g}^{-1} dw$) and the individuals' histo-pathological score.

558 Figure 1
560

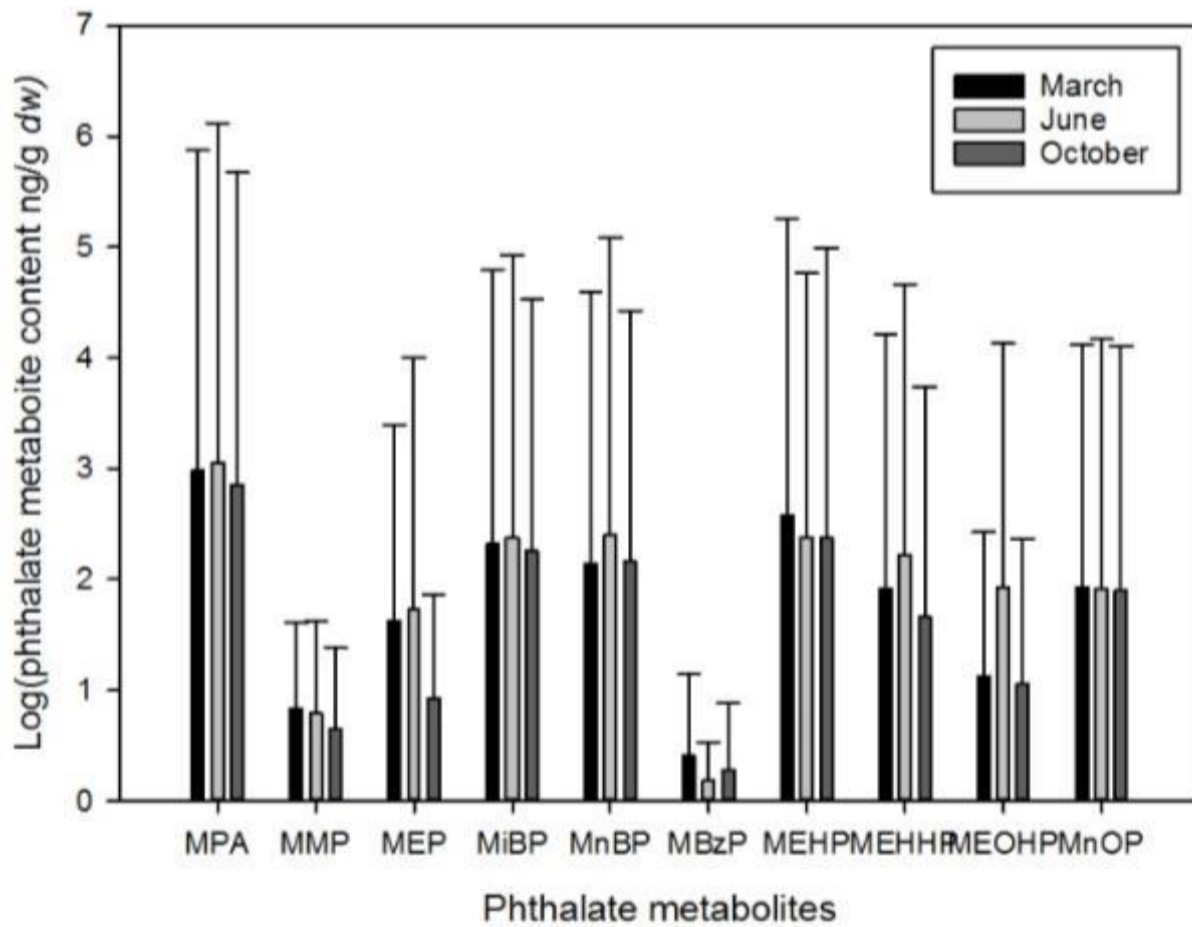


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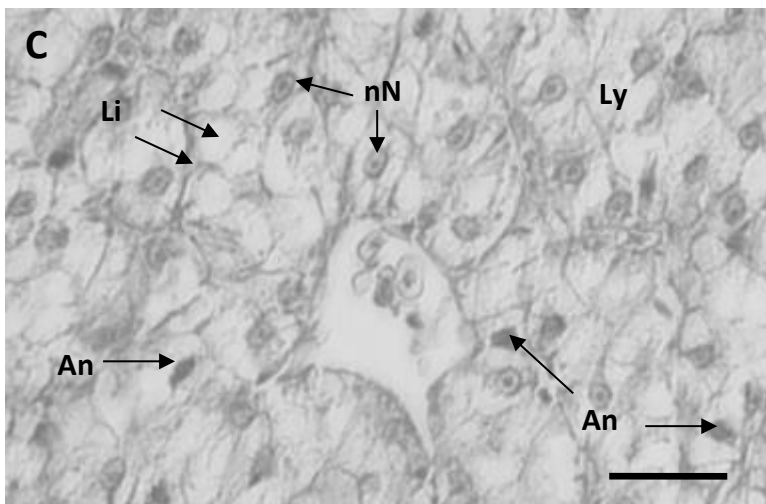
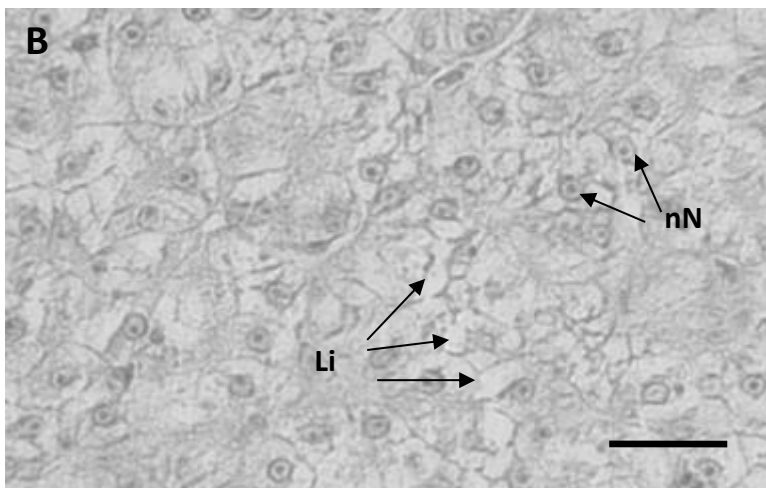
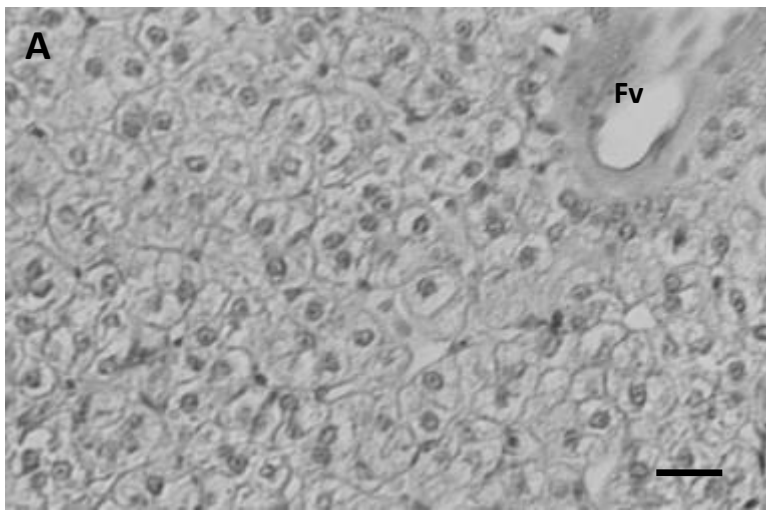
564 Figure 2.

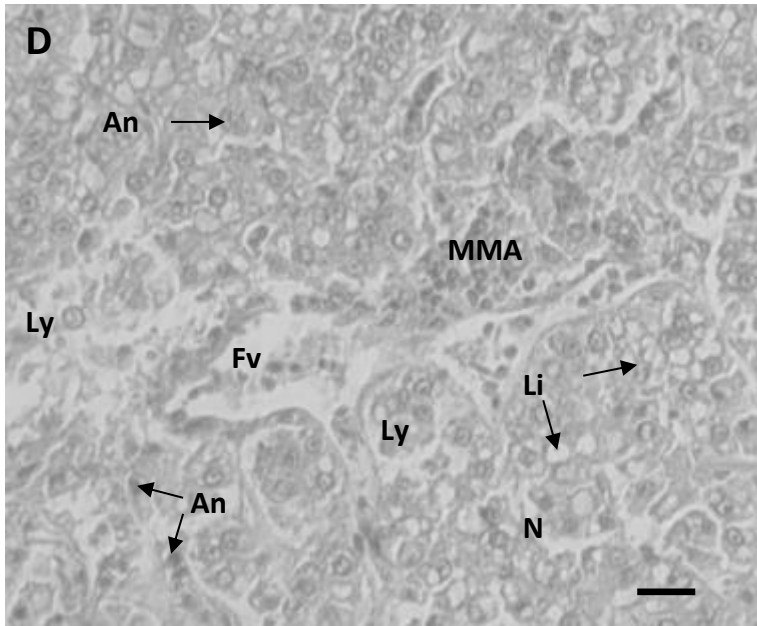
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566

567 Figure 3.
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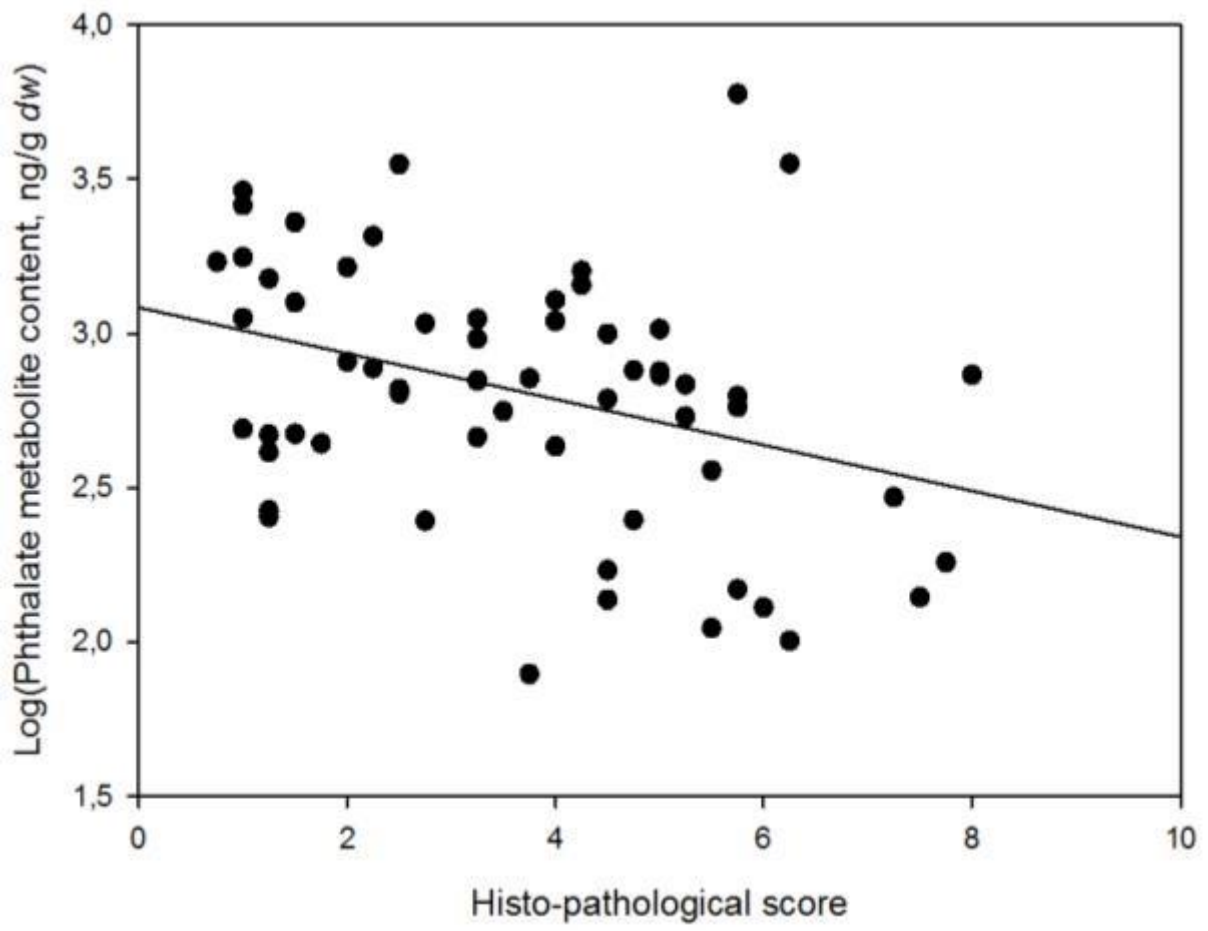
575

576

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578 Figure 4.

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