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1 **Transgenerational metabolic disorders and reproduction defects**
2 **induced by benzo[a]pyrene in *Xenopus tropicalis***

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34 **Abstract**

35 Metabolic disorders induced by endocrine disruptors (ED) may contribute to amphibian
36 population declines but no transgenerational studies have evaluated this hypothesis. Here
37 we show that *Xenopus tropicalis*, exposed from the tadpole stage, to the ED benzo[a]pyrene
38 (BaP, 50 ng.L⁻¹) produced F2 progeny with delayed metamorphosis and sexual maturity. At
39 the adult stage, F2-BaP females displayed fatty liver with inflammation, tissue
40 disorganization and metabolomic and transcriptomic signatures typical of nonalcoholic
41 steato-hepatitis (NASH). This phenotype, similar to that observed in F0 and F1 females, was
42 accompanied by a pancreatic insulin secretory defect. Metabolic disrupted F2-BaP females
43 laid eggs with metabolite contents significantly different from the control and these eggs did
44 not produce viable progeny. This study demonstrated that an ED can induce
45 transgenerational disruption of metabolism and population collapse in amphibians under
46 laboratory conditions. These results show that ED benzo[a]pyrene can impact metabolism
47 over multiple generations and support epidemiological studies implicating environmental EDs
48 in metabolic diseases in humans.

49

50

51 **Capsule abstract**

52 Benzo[a]pyrene can induce transgenerational disruption of metabolism and population
53 collapse in amphibians under laboratory conditions.

54

55

56 **Keywords:** endocrine disruptor, transgenerational, metabolic syndrome, amphibian
57 population decline, *Xenopus tropicalis*

58

59 **1. Introduction**

60 Amphibians are one of the most impacted vertebrate group based on population declines
61 with least at 41% of all known amphibian species are threatened with extinction (IUCN,
62 2019). Reasons for this decline include habitat loss, introduction of exotic species and
63 emerging infectious diseases. Pollution of wetland habitats by endocrine disruptors (ED) may
64 also be involved (Carey et al., 1999; Hayes et al., 2010a; Kiesecker, 2002). An endocrine
65 disruptor is an exogenous substance or mixture that alters the function(s) of the endocrine
66 system and consequently causes adverse health effects in an intact organism, or its progeny,
67 or in (sub) populations (WHO, 2012). Field studies have suggested that ED may contribute to
68 population decline in amphibians by altering population fitness (Fedorenkova et al., 2012)
69 and amphibians might act as environmental sentinels (Hayes et al., 2010a). ED can interfere
70 with the crosstalk between the thyroid and the reproductive axes in amphibians (Duarte-
71 Guterman et al., 2014). ED can also disrupt organogenesis and gonadal differentiation in
72 larvae (Crump et al., 2002; Hammond et al., 2015; Hayes et al., 2010b; Helbing, 2012;
73 Langlois et al., 2010; Opitz et al., 2005) and egg and sperm maturation in adults (Hayes et
74 al., 2011; Hayes et al., 2006; Hayes et al., 2002; Hayes et al., 2010b; Orton and Tyler, 2015;
75 Safholm et al., 2014; Urbatzka et al., 2007). In addition their involvement in lasting alterations
76 of immunity and resistance to infectious pathogens has been also highlighted (Robert et al.,
77 2018; Robert et al., 2019). Studies on humans and other mammals suggest that metabolic
78 disorders might be associated with ED (Casals-Casas and Desvergne, 2011; Chevalier and
79 Fenichel, 2015, 2016). However, the involvement of ED exposure in the metabolic disorders
80 associated with amphibian population decline has received little study. Initial evidence that
81 ED can alter frog metabolism was documented in *Xenopus tropicalis* after acute exposure to
82 triclosan and benzo[a]pyrene (BaP) (Regnault et al., 2016; Regnault et al., 2014). Controlled
83 exposures of *X. tropicalis* throughout their life cycle to these two ED, alone or in mixtures at
84 environmentally relevant concentrations, induced a metabolic syndrome featuring a pre-
85 diabetic state. The treated frogs produced progeny with slower development, smaller adult
86 size, and reduced reproductive success. These results link metabolic disorders to putative

87 population declines and highlight the potential multigenerational impacts of ED (Regnault et
88 al., 2018; Usal et al., 2019).

89 Studies in mammals and fishes show that ED may cause multi- and transgenerational effects
90 leading to reprotoxicity, cancer, metabolic impairments, obesity, diabetes, and
91 neurodegeneration in progeny that were not directly exposed (Chen et al., 2019a; Coimbra et
92 al., 2015; DeCourten et al., 2019; Perera et al., 2020; Skinner, 2014; Xie et al., 2019). The
93 transmission of ED effects across generations may result from epigenetic mechanisms
94 (Baker et al., 2014; Major et al., 2020; Perera et al., 2020). However, in many species, it is
95 believed that healthy offspring are born by a healthy mother (Swain and Nayak, 2009) since
96 offspring sustainability is dependent on female investment in the egg yolk (Hedayatirad et al.,
97 2020). Therefore, detrimental maternal effects on offspring could be explained by a lower
98 investment in egg reserves (Hedayatirad et al., 2020). Metabolic impairment of amphibian
99 females exposed to ED could affect progeny during several generations and the
100 transgenerational effects of ED could be a component of amphibian population declines.

101 We investigated the transgenerational impact of BaP on *Xenopus* females following F0
102 generation exposure from the tadpole to the mature adult stage at a concentration (50 ng.L⁻¹)
103 considered to be a safe level for drinking water (Health Canada, 2016; WHO, 2003).. BaP
104 was chosen because it is a widespread contaminant, classified as ED (USPEA; Petersen et
105 al., 2007) and it can markedly affect amphibian metabolism at the concentration used in this
106 study (Regnault et al., 2018). In addition, Ah-R ligands like BaP have been shown to induce
107 transgenerational effects in fish (Baker et al., 2014). In contrast to mammals, amphibian eggs
108 are fertilized in water and embryos develop externally. During BaP exposure of the parental
109 F0 generation, the F1 progeny are exposed only at the gamete stage. The gametes
110 producing the F2 frog generation are not exposed so the effects seen in F2 amphibians are
111 transgenerational (Baker et al., 2014). Although the purpose of this study was to investigate
112 the transgenerational impacts of BaP on the F2, putative metabolic disorders were observed
113 in F1 and F2 females to determine the phenotype transmission from F0 to F2 progeny. Here

114 we showed that this widespread ED can induce severe multi-and transgenerational metabolic
115 disorders in female *Xenopus* leading to a total reproductive disruption at the F2. These data
116 suggest that ED acting as metabolic disruptors may have a direct causal relationship with
117 amphibian population decline and provide proof of their transgenerational effects in this
118 phenomenon.

119 **2. Material and methods**

120 *2.1. Ethics statement*

121 All experiments were performed in an accredited animal house (LECA, n° C3842110001)
122 and approved by the ethics committee ComEth Grenoble - C2EA – 12 (animal welfare
123 agreement n° 02545.03).

124

125 *2.2. Animals*

126 F0 animals were exposed to 50 ng.L⁻¹ BaP from the seven-day old tadpole stage to mature
127 adult stage as previously described (Regnault et al., 2018). This concentration matched BaP
128 concentrations often found in polluted waters (Olivares-Rubio et al., 2015; Trapido and
129 Ingeborg, 1996), and is below the maximum acceptable safe drinking water concentration
130 (Health Canada, 2016; WHO, 2003). At the adult stage, five F0 females and ten F0 males
131 were selected from the BaP treatment and from the control treatment and allowed to mate.
132 The methods used are described in the SI.

133

134 *2.3. Glucose tolerance test*

135 Frogs were fasted 24 hours before the test. Glycaemia was measured by digital sampling at
136 0 and 8 hours after intraperitoneal glucose injection (1 mg.g⁻¹ fresh mass) using a glucometer
137 (Accu-Chek Performa, Roche Diagnostics, Meylan, France).

138

139 *2.4. Insulin resistance test*

140 Frogs were fasted 12 hours before the test. Glycaemia was measured by digital sampling at
141 0 and 5 hours after injection of bovine insulin (Sigma Aldrich, France) in dorsal lymph sacs (5
142 mU.g⁻¹ fresh mass) using a glucometer (Accu-Chek Performa, Roche Diagnostics, Meylan,
143 France).

144

145

146

147 *2.5. Histological observations*

148 The liver (same lobe/frog), the pancreas and the muscle were collected, embedded in Tissue
149 OCT (Labonord - VWR, Templemars, France), frozen in liquid nitrogen, and stored at -80°C.
150 Using a cryostat (CM3050 S, Leica, Nussloch, Germany), histological transverse sections 7
151 µm thick were produced and mounted on Super-Frost plus microscope slides (Labonord).
152 Staining procedures are described in the SI.

153

154 *2.6. Plasma insulin concentration measurements*

155 Insulinemia was measured in 24 hours-fasting F2 females or after a glucose challenge. The
156 methods used are described in the SI.

157

158 *2.7. Statistics*

159 For F1 and F2 females, phenotypic data are expressed as the mean ± SEM derived from 6 to
160 16 individual experiments, depending on the measured parameter (see Fig. legends). Since
161 the statistical distribution of the data was Gaussian for several data and not for other, a
162 Wilcoxon's test was used to compare the BaP-exposure population to the control population.
163 To statistically compare the percentage of F2 females showing pancreatic inflammation from
164 F2 control and F2-BaP populations a χ^2 test was used.
165 The distributions of animal development times (age to metamorphosis) and female maturity
166 time for the F2 generation were compared between the control populations and the BaP
167 population using a Kaplan-Meyer's test.

168

169 *2.8. Liver RNA extraction and sequencing*

170 For each biological replicate, the RNAqueous®-4PCR Kit (Ambion, Austin USA) was used to
171 extract the total RNA from 15 mg of liver according to manufacturer instructions. RNA-seq
172 libraries were prepared using the TruSeq Stranded mRNA sample Prep kit (Illumina, San
173 Diego, USA) and sequenced on an Illumina GAIIx sequencer as 75 bp reads by Hybrigenics-

174 Helixio (Clermont-Ferrand, France). The methods used for sequencing analysis are
175 described in the SI.

176

177 *2.9. Liver and egg metabolomics*

178 Liver and egg samples (20-50 mg) were extracted in methanol/water/dichloromethane
179 (2/1.4/2 v/v/v) using the protocol described by Beckonert et al. (Beckonert et al., 2007). The
180 methods used for metabolomics analysis are described in the SI.

181

182 **3. Results**

183 *3.1. Parental exposure leads to metabolic disruption in F1 progeny*

184 The main goal of this paper was to determine the transgenerational impacts of BaP but
185 putative metabolic disorders were also studied in F1 females to assess phenotype
186 transmission from the F0 generation to F2 progeny. No difference in hepatosomatic and
187 adiposomatic indexes were found in F1-BaP females despite a marked increase of liver lipid
188 contents (1.7 fold, $p=0.003$) (Fig. 1a-c). In addition to steatosis, the liver sections of F1-BaP
189 females showed hepatocytes with fewer cell contacts and irregular shapes, leukocyte
190 infiltrates, and swollen blood vessels typical of a progression from nonalcoholic fatty liver
191 disease (NAFLD) to nonalcoholic steatohepatitis (NASH) (Fig. 1d-g). For carbohydrate
192 metabolism, no difference in basal glycaemia was found in F1-BaP females despite a
193 marked decrease of fasting plasma insulin concentrations (0.4 fold, $p=0.008$) (Fig. 1h, i).
194 However, no decrease in liver and muscle carbohydrate content was observed (Fig. 1j, k). To
195 study the molecular mechanism associated with metabolic disruption, the liver transcriptomic
196 signature of F1-BaP females was compared to control frogs. Liver transcriptomes of F1-BaP
197 females showed differential transcription of 130 genes with down-regulation in 70% of cases.
198 (Table S1). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment
199 analyses of the differentially transcribed genes indicated a link between BaP-parental
200 exposure and "Steroid biosynthesis" (40.4-fold, $p=1.12 \text{ E-}11$), "Biosynthesis of antibiotics"
201 (6.6-fold, $p=1.36 \text{ E-}8$), "Terpenoid backbone biosynthesis" (22.5-fold, $p=5.22 \text{ E-}5$), "Metabolic

202 pathways” (2-fold, p=1.09 E-4), “Pyruvate metabolism” (9.5-fold, p=0.007), “PPAR signaling
203 pathway” (6.5-fold, p=0.02). General ontology (GO) terms enrichment analyses of the
204 differentially transcribed genes indicated a link between BaP-parental exposure and “Sterol
205 biosynthetic process” (64.9-fold, p=8.29 E-4), “Isoprenoid biosynthetic process” (43.3-fold,
206 p=0.0019), “Response to yeast” (27.8-fold, p=0.0048), “Iron ion binding” (4.8-fold, p=0.018)
207 (Fig. S1). These enriched pathways indicate global metabolic impairment encompassing
208 lipid, carbohydrate and protein metabolism. However, to compare metabolic impairment
209 occurring in F1 and F2 females, we manually assigned each gene to general pathways
210 known to be affected in NAFLD or NASH pathology (Fig. 1I). Many differentially transcribed
211 genes were associated with lipid metabolism and highlighted a marked downregulation of
212 genes involved in cholesterol metabolism (from 0.06 to 0.45-fold) including *3-hydroxy-3-*
213 *methylglutaryl-CoA reductase (hmgcr)*, *3-hydroxy-3-methylglutaryl-CoA synthase 1*
214 *(hmgcs1)*, *isopentenyl-diphosphate delta isomerase 1 (idi1)*, *methylsterol monooxygenase 1*
215 *(msmo1)*, *cytochrome b5 reductase 2 (cyb5r2)*, *acetyl-CoA acetyltransferase 2 (acat2)*,
216 *mevalonate diphosphate decarboxylase (mvd)*, *squalene epoxidase (sqle)*, *lanosterol*
217 *synthase (lss)*, *7-dehydrocholesterol reductase (dhcr7)*, *hydroxysteroid (17-beta)*
218 *dehydrogenase 7 (hsd17b7)*, *sterol-C5-desaturase (sc5d)*, *24-dehydrocholesterol reductase*
219 *(dhcr24)*, *cytochrome P450 51A1 (cyp51a1)*, *cytochrome P450 7A1 (cyp7a1)* and *NAD(P)*
220 *dependent steroid dehydrogenase-like (nsdhl)* (Fig. 1I). A large number of genes involved in
221 fatty acid metabolism (*fatty acid amide hydrolase, faah.1*; *acyl-CoA synthetase 2.2, acss2.2*;
222 *lysophosphatidylglycerol acyltransferase 1, lpgat1*; *lipase, hormone-sensitive, lipe*) and
223 transport (*solute carrier family 27 member 4, slc27a4*) were downregulated. Only the gene
224 encoding for solute carrier family 27 member 6 (*slc27a6*), involved in long chain fatty acid
225 transport across membranes was upregulated (2.43-fold) (Fig. 1I). Analysis of genes involved
226 in carbohydrate metabolism and insulin signaling pathways showed activation of
227 *phosphoenolpyruvate carboxykinase 1 (pck1)*, 36.4-fold) and *insulin like growth factor binding*
228 *protein 1 (igfbp1)*; 5.28-fold) genes (Fig. 1I). Among the genes involved in protein metabolism,
229 there was a marked deregulation of the genes encoding HSP 70 and HSP 90 proteins (0.18

230 and 0.35-fold) suggesting endoplasmic reticulum stress (ER-stress). ER-stress has been
231 associated with cell death and apoptosis (Faitova et al., 2006). We found that *DNA damage*
232 *inducible transcript 4 (ddit4)* and *protein tyrosine phosphatase 4A1 (ptp4a1)* genes that are
233 involved in the activation of cell death during stress (Chen et al., 2016; Halle et al., 2007)
234 were upregulated (3.86 and 2.42-fold) (Fig. 1l). Analysis of genes associated with the
235 immune system confirmed the histological data indicating an inflamed liver condition (Fig. 1d-
236 g). Indeed, genes promoting inflammation (*signal transducer and activator of transcription 4,*
237 *stat4; macrophage scavenger receptor 1, msr1; DEXD/H-box helicase 58, ddx58 and*
238 *interleukin 18 binding protein –like, il18bp*) were upregulated (Fig. 1l). Thus, a histological
239 phenotype highlighting decreased cell-cell contact was confirmed by the downregulation of
240 genes involved in tight-junction integrity including *cadherin related family member 4 (cdhr4,*
241 *0.25-fold)* and *F11 receptor (f11r, 0.35-fold)* genes (Fig. 1l).

242

243 *3.2. F0-exposure to BaP leads to transgenerational impairment of F2 development and* 244 *metabolism.*

245 The F2 progeny from F0 frogs exposed to BaP had significantly delayed metamorphosis ($p=2$
246 $E-16$) (the 50% population metamorphosis level was reached 20 days later) compared to the
247 control (Fig. 2a). In addition, F2 females from BaP exposed animals also showed delayed
248 sexual maturity ($p= 8 E-04$), taking more than 80 days to reach 80% of mature *Xenopus* in
249 the population compared to the controls (Fig. 2b). Analysis of metabolic disorders in sexually
250 mature F2-BaP females showed increased hepatosomatic (1.2 fold, $p=0.043$) and
251 adiposomatic (1.8 fold, $p=0.026$) indexes (Fig 2c, d). The liver sections of F2-BaP females
252 had hepatocytes with fewer cell contacts and irregular shapes, leukocyte infiltrates, and
253 swollen blood vessels (Fig. 2e-h). In carbohydrate metabolism, fasting glycaemia was
254 increased in F2-BaP females compared to the controls without modification of fasting plasma
255 insulin concentrations (Fig. 2i, j). To determine if the increase in fasting glycaemia in F2-BaP
256 females was due to default in cell glucose assimilation, we performed glucose tolerance
257 tests. Intraperitoneal injection of glucose led to a higher increase in the blood glucose level of

258 F2-BaP females compared to the controls. At 6-hours post-glucose injection, glycaemia of
259 F2-BaP females was about 1.4 times that of controls ($p=0.008$) (Fig. 2k). This glucose
260 intolerance was accompanied by a decrease in muscle glycogen stores (Fig. 2l). To
261 determine if the observed-glucose intolerance and the subsequent decrease in carbohydrate
262 contents in muscle was linked to insulin resistance, we monitored glycaemia after insulin
263 injection. Interestingly, F2-BaP females did not display insulin resistance (Fig. 2m). However,
264 by monitoring plasmatic insulin concentrations after glucose injection, we found a marked
265 reduction in insulin secreting capacities of F2-BaP females (0.5 fold, $p=0.005$). Plasmatic
266 insulin concentrations, measured 30 minutes after glucose injection, were 2.2-times lower in
267 F2-BaP females than in the controls (Fig. 2n). **At the histological level, a non-significant**
268 **decrease ($p=0.57$) in insulin production by the pancreatic β -cells was observed between F2-**
269 **BaP females comparatively to controls** (Fig. S2). However, pancreatic sections revealed an
270 increase in leukocyte infiltrates in F2-BaP females compared to the controls (Fig. 2o).

271

272 *3.3. Liver transcriptomic signature of F2-BaP females confirms metabolic impairments.*

273 To study the molecular mechanisms associated with metabolic disruption, the liver
274 transcriptomic signature of F2-BaP females was compared to controls. Liver transcriptomes
275 of F2-BaP females showed differential transcription of 104 genes with upregulation in 54% of
276 cases (Table S2). KEGG pathway enrichment analyses of the differentially transcribed genes
277 indicated a link between BaP-F0 exposure and “Steroid biosynthesis” (29.9-fold, $p=1.58 \text{ E-}5$)
278 and “Biosynthesis of antibiotics” (5.3-fold, $p=1.58 \text{ E-}4$). GO-terms enrichment analyses of the
279 differentially transcribed genes indicated a link between BaP-F0 exposure and “Regulation of
280 cell growth” (18.8-fold, $p=0.01$), “Sterol biosynthetic process” (48.1-fold, $p=0.04$), and
281 “Establishment of protein localization to plasma membrane” (48.1-fold, $p=0.04$) (Fig. S3). We
282 manually assigned each gene to general pathways known to be affected in NAFLD or NASH
283 pathology (Fig. 3a). Many differentially transcribed genes were associated with lipid
284 metabolism and there was a marked upregulation of genes involved in cholesterol
285 metabolism including *3-hydroxy-3-methylglutaryl-CoA reductase (hmgcr, 2.58-fold)*;

286 *hydroxysteroid (17-beta) dehydrogenase 7 (hsd17b7, 1.98-fold), cytochrome P450 51A1*
287 *(cyp51a1, 3.35-fold), farnesyl-diphosphate farnesyltransferase 1 (fdft1, 2.07-fold),*
288 *methylsterol monooxygenase 1 (msmo1, 4.98-fold) and squalene epoxidase (sqle, 14.34-*
289 *fold) (Fig. 3a). In addition, two genes involved in fatty acid and triglyceride metabolism (1-*
290 *acylglycerol-3-phosphate O-acyltransferase 4, agpat4) and transport (solute carrier family 27*
291 *member 3, slca27a3) were downregulated. Transcriptomic data also revealed upregulation of*
292 *genes encoding the steatosis protective proteins regulocalcin (rgn, 2.38-fold), sulfotransferase*
293 *2B1 (sult2b1, 2.27-fold) and lipase hormone-sensitive (lipe, 9.33) (Fig. 3a). Analysis of genes*
294 *involved in carbohydrate metabolism and insulin signaling pathways revealed increased*
295 *activation of insulin like growth factor binding protein 1 gene (igfbp1; 2.86-fold) (Fig. 3a). In*
296 *addition, nuclear receptor 4A1 gene (nr4a1) involved in gluconeogenesis activation was*
297 *downregulated (0.25-fold) together with upregulation of prolactin regulatory element binding*
298 *gene (preb, 1.96-fold) known to inhibit liver glucose production (Fig. 3a). Compared to F1-*
299 *BaP females, transcriptomic data did not reveal ER-stress or cell death markers in F2-BaP*
300 *females. All genes involved in cell death/proliferation appeared to be strongly under-*
301 *transcribed (Fig. 3a). Analysis of genes associated with the immune system confirmed the*
302 *histological data and suggested an inflammatory liver state (Fig. 2e-h). Genes promoting*
303 *inflammation appeared upregulated including C-reactive protein 4, (crp4, 4.09-fold),*
304 *eosinophil peroxidase (epx, 2.39-fold) and mitogen-activated protein kinase 8 (map3k8, 2.70-*
305 *fold) (Fig. 3a). Thus, a histological phenotype highlighting decreased cell-cell contact was*
306 *confirmed by the marked downregulation of cadherin 10 (cdh10, 0.26-fold) and claudin 15.1*
307 *(cldn15.1, 0.33-fold) genes involved in tight-junction integrity (Fig. 3a).*

308

309 *3.4. Liver metabolomic signature of F2-BaP females indicates impairment of amino acids,*
310 *lipids and carbohydrate metabolism.*

311 To compare the metabolic profiles of the liver from F2-BaP and control females, partial least
312 squares-discriminant analysis (PLS-DA) was performed using the ¹H NMR spectra of the
313 aqueous liver extracts. A PLS-DA model was then fitted on the filtered and centered data. A

314 valid and robust model was adjusted ($A=2$; $R^2=97.3\%$; $Q^2=0.89$). The score plot showed
315 clear discrimination between control and F2-BaP samples on the first axis (Fig. S4a). We
316 found 17 discriminant metabolites between F2-BaP and control females (Fig. 3b). The levels
317 of branched amino acids (leucine, Isoleucine and valine), aromatic amino acids
318 (phenylalanine and tyrosine), acidic amino acid (glutamate) as well as threonine, proline and
319 alanine were decreased in F2-BaP females. In addition, creatine and glutathione contents
320 were decreased by 23.3% ($p=0.024$) and 20.9% ($p=0.014$), respectively, in F2-BaP females.
321 Glucose contents were decreased by 14.3% ($p=0.034$) and glycogen contents increased by
322 48.1% ($p=0.017$) in F2-BaP females compared to controls. Formate and acetate contents
323 were increased by 97.3% ($p=0.024$) and 52.6% ($p=0.024$) in F2-BaP females. Choline, an
324 important component of the cell membrane, was significantly reduced (-16.6%, $p=0.024$) in
325 F2-BaP females.

326 For liver lipid extracts, a PLS-DA model was fitted on filtered and Pareto-scaled data. A valid
327 and robust model was adjusted ($A=3$; $R^2=0.906$; $Q^2=0.739$). The score plot showed clear
328 discrimination between control and F2-BaP samples on the first axis (Fig. S4b). Triglyceride
329 contents were increased by 19.91% ($p=0.014$) in F2-BaP females suggesting steatosis
330 development. This phenomenon was confirmed by the increase of CH_3 fatty acid (1.56%,
331 $p=0.04$), CH_2 fatty acids (3.16%, $p=0.018$) and unsaturated fatty acid (3.63%, $p=0.023$)
332 contents in F2-BaP liver. In contrast, total cholesterol and free cholesterol were, respectively,
333 decreased by 12.9% ($p=0.007$) and 10.6% ($p=0.007$) in F2-BaP females compared to the
334 controls (Fig. 3b).

335

336 ***3.5. Transgenerational metabolic disruption of F2-BaP *Xenopus* is associated with*** 337 ***reproductive defects.***

338 We evaluated the gonadosomatic index as a proxy for the female body reserve investment
339 into gonadal development. F2-BaP females had a decreased gonadosomatic index
340 compared to controls (0.8 fold, $p=0.035$) (Fig. 4a). To compare the metabolic profiles of the
341 eggs from F2-BaP and control females, partial least squares-discriminant analysis (PLS-DA)

342 was performed based on the ^1H NMR spectra of the aqueous and lipids extracts. PLS-DA
343 models were then fitted on filtered and centered data. Two valid and robust models were
344 adjusted for aqueous ($A=3$; $R^2=0.342$; $Q^2=0.699$) and lipid extracts ($A=3$; $R^2=0.984$;
345 $Q^2=0.924$). The score plots showed a clear discrimination between control and F2-BaP egg
346 samples on the first axis (Fig. S5a, b). Analysis of discriminative metabolites in aqueous
347 extracts revealed a decrease in glutamine, histidine and tryptophan amino acids together
348 with creatine and glutathione contents in eggs from F2-BaP females (Fig. 4b). In addition,
349 citrate and ATP/ADP/AMP contents were also decreased, respectively, by 8.8% ($p=0.02$) and
350 10.4% ($p=0.025$) in eggs from F2-BaP females. Among the aqueous metabolites, only
351 butyrate increased (11.7%, $p=0.033$) in F2-BaP eggs (Fig. 4b). Analysis of discriminative lipid
352 metabolites in F2-BaP eggs revealed decreased total cholesterol (-7.7%, $p=0.0016$),
353 cholesterol ester (-8.6%, $p=0.0073$), free cholesterol (-5.3%, $p=0.0016$) and CH_3 fatty acids (-
354 0.7, $p=0.017$) contents and a slight increase in triglyceride, CH_2 fatty acids, and
355 monounsaturated fatty acid contents. Phospholipid contents slightly decreased by 4.8%
356 ($p=0.0053$) in eggs from F2-BaP females compared to controls (Fig. 4b).
357 To assess F2 reproductive success, we mated five F2 pairs from both the control and F2-
358 BaP group. Only 60% (3) of the F2-BaP females laid eggs compared to 100% (5) of the
359 controls following stimulation (Fig. 4c). In addition, whereas amplexus was successful for
360 100% of the controls, it was successful for only 40% (2) of F2-BaP frogs (Fig. 4d). F2 mating
361 of control frogs produced an average of 270 hatched eggs per mating pair. In contrast, no
362 viable larvae were obtained from the F2-BaP frogs (Fig. 4e).

363

364 **4. Discussion**

365 Experimental and epidemiological evidence from humans and other mammals has
366 demonstrated a correlation between ED exposure and metabolic diseases (Casals-Casas
367 and Desvergne, 2011). Studies using rodent models have highlighted the potential of ED to
368 cause multigenerational and transgenerational metabolic effects (Lee and Blumberg, 2019).
369 However, the potential involvement of ED in metabolic impairments as a possible cause of

370 amphibian population decline has been largely neglected. We studied controlled exposures
371 of *X. tropicalis*, throughout their life cycle, to the ED BaP at environmentally relevant
372 concentrations such as could be found in “safe” drinking water (Health Canada, 2016; WHO,
373 2003). These levels of BaP induced a metabolic syndrome in *X. tropicalis* featuring a pre-
374 diabetic state (Regnault et al., 2018). In the present study, we highlighted that parental
375 exposure to BaP, can cause multigenerational and transgenerational metabolic impairments
376 in *Xenopus* and reproductive failure in the F2 generation.

377 F1-BaP females, only exposed at the gamete stage, displayed marked liver steatosis
378 associated with liver inflammation, swollen blood vessels and tissue integrity loss typical of a
379 progression between NAFLD to NASH (Takahashi and Fukusato, 2014) (Fig. 1c-g).

380 Inflammatory phenotype was confirmed by the upregulation of genes promoting inflammation
381 (*stat4; msr1; ddx58 and il18bp*) and tissue integrity loss by the marked deregulation of genes
382 involved in a tight junction (*cdhr4, and f11r*) (Fig. 1I). This phenotype could be induced
383 through aryl hydrocarbon receptor (Ah-R) activation, since this nuclear receptor is involved in
384 NAFLD/NASH and liver inflammatory processes in mice (Moyer et al., 2016). **The F1**
385 **generation was exposed at the gamete which constitute critical time windows to induce**
386 **epigenetic alterations across multiple generations.** Recent findings have provided evidence
387 of Ah-R activation in mediating these effects (Viluksela and Pohjanvirta, 2019).

388 Multigenerational action of Ah-R is confirmed by the marked inhibition of genes involved in
389 cholesterol metabolism. Ah-R can modulate the expression of a number of genes involved in
390 the cholesterol synthesis pathway in mammals among which *hmgcr, hsd17b7, cyp51a1, and*
391 *sqle* were deregulated in F1-BaP females (Tanos et al., 2012) (Fig. 1I). Ah-R induced
392 steatosis is also associated with suppression of fatty acid oxidation (Lee et al., 2010) and F1-
393 BaP females displayed a markedly decreased transcription of the *lipo* gene encoding a
394 critical enzyme involved in triglyceride degradation and fatty acid oxidation (Reid et al.,
395 2008). Fatty liver disease is associated with endoplasmic reticulum stress (ER stress) and
396 apoptosis (Bechmann et al., 2012; Naik et al., 2013). We showed that F1-BaP females had a
397 marked deregulation of genes coding for proteins involved in adaptive unfolded protein

398 response (UPR) (*hsp70* and *hsp90*) (Vincenz et al., 2013) together with genes involved in the
399 activation of cell death during stress (*ddit4* and *ptp4a1*) (Chen et al., 2016; Halle et al., 2007)
400 (Fig. 1l). ER stress is a primary event of insulin resistance development (Otoda et al., 2013).
401 No difference in carbohydrate contents in muscle was observed suggesting the absence of
402 muscle insulin resistance (Fig. 1 j). However, despite no difference in basal glycaemia (Fig. 1
403 h), transcriptomic data in liver revealed activation of the *pck1* gene (Fig. 1l) which could be
404 linked to hepatic insulin resistance or insulinopenia (Bechmann et al., 2012; Gomez-Valades
405 et al., 2008). However, the marked activation of *igfbp1*, associated with insulinopenia
406 (Brismar et al., 1994), and the marked decrease of fasting plasma insulin concentrations
407 (Fig. 1i) suggests a pancreatic insulin secretory defect. Overall, our results demonstrate that
408 the phenotype observed in F1-BaP females was similar to that of F0 animals exposed during
409 their entire life to BaP (Regnault et al., 2018). This suggests that multigenerational
410 transmission of metabolic impairments can occur in frogs.

411 As previously stated, the major goal of this paper was to investigate the transgenerational
412 metabolic impacts of BaP and their potential consequence on fitness. F2-BaP frogs had
413 significantly delayed metamorphosis (an increase of 20 d for 50% of the population to reach
414 frog metamorphosis) (Fig. 2a) similar to the F0 and F1 generations (Regnault et al., 2018).
415 This demonstrated the transgenerational activity of this ED. These types of disturbances
416 could play a major role in the amphibian population decline as the delayed metamorphosis
417 reduces adult recruitment and reduces the chances for successful reproduction (Smith,
418 1987). Our data clearly showed that delayed metamorphosis in females was accompanied by
419 delayed sexual maturity, taking more than 80 days to reach 80% of mature *Xenopus* in the
420 population compared to the controls (Fig. 2b). Delayed sexual maturity of females was
421 accompanied by reduced developmental capabilities of their progeny which would probably
422 decrease their chances of survival in case of pond drying (Gomez-Mestre et al., 2013).
423 Metabolic disorders were studied in sexually mature females. Despite having different
424 regulation of genes associated with carbohydrate and lipid metabolism than that observed in

425 F0 and F1 animals, the underlying mechanisms also seemed to be associated with insulin
426 secretion defects. F2-BaP females had an increased hepatosomatic index associated with a
427 20% increase in liver triglycerides. Liver steatosis associated with triglycerides and fatty acid
428 accumulation suggest the development of NAFLD (Fig. 2c, 3b). This phenotype was
429 confirmed by the marked activation of the genes encoding for regulocalcin, sulfotransferase
430 2B1 and *lipase hormone sensitive* known to protect liver from steatosis (Musso et al., 2013;
431 Reid et al., 2008; Yamaguchi and Murata, 2013) suggesting a fight against lipid
432 accumulation. In F2-BaP females, liver inflammation, swollen blood vessels and loss of
433 tissue integrity highlighted the progression from NAFLD to NASH (Takahashi and Fukusato,
434 2014). This phenotype was confirmed by transcriptomic data showing a 4-fold increase in the
435 transcription level of *crp4*, *epx* and *map3k8* genes. Crp increase in the liver has been
436 associated with NAFLD and diabetes in mammals (Foroughi et al., 2016). In addition,
437 decreased cell-cell contacts was confirmed by the downregulation of *cadherin* and *claudin 15*
438 genes involved in tight-junction integrity (Fig. 3a).

439 An increase in basal serum glucose levels was found between F2-BaP females with no
440 change in insulin levels (Fig. 2i, j) which constitutes a marked difference between F1- and
441 F2-BaP progeny. F2-BaP females also displayed a reduction in glucose tolerance
442 accompanied by a decrease in muscle glycogen stores (Fig. 2k, l). This phenotype was not
443 associated with insulin resistance (Fig. 2m) as suspected in F0-BaP females (Regnault et al.,
444 2018) but to marked insulin secretory capacity defects (Fig. 2n) as suggested by data from
445 F1-BaP females. Transcriptomic data from liver showing a marked upregulation of the *igfbp1*
446 (Brismar et al., 1994) gene confirmed the observed insulinopenia but other transcriptomic
447 and metabolomic markers suggested complex retroaction mechanisms associated with high
448 glycaemia levels. Indeed, metabolomics and transcriptomic data from the liver revealed a
449 decrease in free and total cholesterol levels together with an increase of genes encoding for
450 cholesterol synthesis including the key regulator *hmgcr* (Fig 3a, b). These results confirmed
451 that low hepatocyte cholesterol levels are key activators of cholesterol synthesis (Burg and
452 Espenshade, 2011). Surprisingly after fasting, high plasma glucose levels were associated

453 with liver glucose depletion and increased liver glycogen content (Fig. 3b). Here we can
454 hypothesize that inappropriate neoglucogenesis activation or inhibition of glycogen
455 degradation during fasting may occur in F2-BaP females leading to a reconstruction or a
456 maintenance of glycogen stores. However, this would not explain the higher blood glucose
457 content. This phenotype has been described in mammals experiencing chronic diabetes
458 (Ferrannini et al., 1990). The observed downregulation of *nuclear receptor 4A1* gene (*nr4a1*)
459 (Fig. 3a) involved in gluconeogenesis activation (Pei et al., 2006) might only be associated to
460 retro-control of supra-normal glycogen stores (Wise et al., 1997). Activation of
461 neoglucogenesis in F2-BaP females was also confirmed by liver depletion of amino acids
462 (Fig. 3b) especially alanine which is the major amino acid precursor of glucose in liver and
463 threonine which also contributes to glucose production in liver (Kaloyianni and Freedland,
464 1990). The decrease observed in the other amino acid contents together with glutathione has
465 been described in mammal diabetic models and suggests liver dysfunction (Chen et al.,
466 2019b; Furfaro et al., 2012).

467 BaP induced multi- and transgenerational impairments in carbohydrate, lipid and protein
468 metabolism associated to defects in insulin secretory capacities (Regnault et al., 2018). In
469 female amphibians a large component of liver metabolism is directed to the constitution of fat
470 bodies associated with reproduction or overwintering survival (Fitzpatrick, 1976; Wright,
471 2003). Liver dysfunction is likely to impair these crucial processes. Data from F2-BaP
472 females revealed a marked increase in the adiposomatic index (Fig. 2d) suggesting that the
473 large part of cholesterol or triglyceride contents of the liver had been directed to the fat body.
474 This increase in fat bodies was accompanied by a decrease in the gonadosomatic index in
475 F2-BaP females (Fig. 4a). In sexually mature amphibians, coelomic fat bodies largely support
476 gonadal growth and maturation over somatic tissues and this is supported by an inverse
477 relationship between coelomic fat body size and gonad size (Wright, 2003). In a healthy,
478 sexually mature female amphibian with a normal ovarian cycle, lipids are preferentially
479 allocated to vitellogenesis over deposition into fat depots. Females with large fat bodies
480 therefore highlight a defect in oocyte maturation (Wright, 2003). To study the reproductive

481 capacity of F2-BaP animals compared to the controls, we used semi-natural mating. Despite
482 reaching sexual maturity, F2-BaP females showed a decrease in reproductive investment
483 since only three of five females laid eggs (Fig. 4c). We also observed mating behavior
484 problems in the F2-BaP group since only two of five amplexi were successful compared to all
485 five in the controls (Fig. 4d). F2-control mating produced eggs with variable, but substantial,
486 hatch rates but F2-BaP females did not produce any F3 progeny and had a complete lack of
487 reproductive success (Fig. 4e). Variable hatch rates occur in natural matings of *X. tropicalis*
488 (Showell and Conlon, 2009). However, the same trend was previously observed in F1-BaP
489 frogs but it was less severe because an F2 generation was produced (Regnault et al., 2018).
490 These results suggest that a BaP-F0 exposure can lead to reproductive defects that become
491 progressively worse in successive generations. Exposure of amphibians to a widely
492 distributed ED, such as BaP, under natural conditions might lead to a population decline. In
493 female frogs, a defect in oocyte maturation or metabolite composition may explain the
494 absence of viable progeny. As noted before, large fat bodies suggest a defect in oocyte
495 maturation (Wright, 2003). Metabolomics data demonstrated that the egg composition of F2-
496 BaP females was very different from that of controls (Fig. S5a, b). Analysis of discriminative
497 metabolites in the F2-BaP group showed a decrease of total cholesterol, cholesterol ester
498 and free cholesterol contents (Fig. 4b) which are essential for oocyte maturation. Cholesterol
499 depletion in amphibian oocytes has been linked to oocyte maturation defects and impaired
500 fecundity (Buschiazzo et al., 2011; Buschiazzo et al., 2012; Buschiazzo et al., 2008).
501 Interestingly the metabolite profile variations of F2-BaP eggs matched the principal variations
502 found in the liver especially for lipid contents (Fig. 3b and 4b). It is possible that disruption of
503 lipid metabolism in the liver may lead to inappropriate lipid storage in the fat bodies and
504 ultimately lead to immature oocytes with high lipid levels but low cholesterol contents. In
505 addition, reduced reproductive success might also have a male component. Zebrafish data
506 has shown the capacity of dioxin, another Ah-R ligand, to decrease reproductive success of
507 males transgenerationally (Baker et al., 2014).

508

509 **5. Conclusion**

510 We focused on finding links between BaP exposure and potential amphibian population
511 decline. The widespread BaP can induce severe multi- and transgenerational metabolic
512 disorders in amphibians at concentrations that are still considered safe for human
513 consumption. Parental exposure to BaP can negatively affect the development and fecundity
514 of their progeny over two generations and lead to reproductive failure in the F2 generation.
515 Our findings serve as a starting point for additional work to elucidate the role of ED acting as
516 metabolic disruptors or obesogens as contributing causes of amphibian population decline.
517 Our results demonstrate the transgenerational effects of ED, which may apply to amphibians
518 and also to mammals, including humans.

519

520

521 **Data access**

522 The RNA-seq sequence data reported in this paper have been deposited on NCBI Gene
523 Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo/info/submission.html>
524 (accession n° GSE150911).

525

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538

539

540 **Author contributions.**

541 MR and SR conceived and designed the experiments; MU, SV, MDGC, CR, SS, JBF, MTF,
542 CC, MR and SR performed the experiments; MU, SV, MDGC, CR, MTF, CC, MR and SR
543 analyzed the data. SR wrote the MS.

544

545 **Competing interests**

546 The authors declare no competing interests.

547 **References**

- 548 U.S. Environmental Protection Agency (USPEA), EDSP21 Dashboard - Endocrine Disruptor
549 Screening Program for the 21st century. Available at <https://actor.epa.gov/edsp21/>
- 550 Baker, T.R., et al., 2014. Dioxin induction of transgenerational inheritance of disease in
551 zebrafish. *Molecular and cellular endocrinology* 398, 36-41.
552 doi:10.1016/j.mce.2014.08.011.
- 553 Bechmann, L.P., et al., 2012. The interaction of hepatic lipid and glucose metabolism in liver
554 diseases. *Journal of hepatology* 56, 952-964. doi:10.1016/j.jhep.2011.08.025.
- 555 Beckonert, O., et al., 2007. Metabolic profiling, metabolomic and metabonomic procedures
556 for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nature protocols* 2,
557 2692-2703. doi:10.1038/nprot.2007.376.
- 558 Brismar, K., et al., 1994. Effect of insulin on the hepatic production of insulin-like growth
559 factor-binding protein-1 (IGFBP-1), IGFBP-3, and IGF-I in insulin-dependent diabetes.
560 *The Journal of clinical endocrinology and metabolism* 79, 872-878.
561 doi:10.1210/jcem.79.3.7521354.
- 562 Burg, J.S., Espenshade, P.J., 2011. Regulation of HMG-CoA reductase in mammals and
563 yeast. *Progress in lipid research* 50, 403-410. doi:10.1016/j.plipres.2011.07.002.
- 564 Buschiazzo, J., et al., 2011. Nongenomic steroid- and ceramide-induced maturation in
565 amphibian oocytes involves functional caveolae-like microdomains associated with a
566 cytoskeletal environment. *Biology of reproduction* 85, 808-822.
567 doi:10.1095/biolreprod.110.090365.
- 568 Buschiazzo, J., et al., 2012. Cholesterol depletion affects the biophysical state of oocyte
569 membranes disturbing amphibian fertilization. *Cryobiology* 65, 359.
- 570 Buschiazzo, J., et al., 2008. Inhibition of *Bufo arenarum* oocyte maturation induced by
571 cholesterol depletion by methyl-beta-cyclodextrin. Role of low-density caveolae-like
572 membranes. *Biochimica et biophysica acta* 1778, 1398-1406.
573 doi:10.1016/j.bbamem.2008.03.004.
- 574 .

575 Carey, C., et al., 1999. Amphibian declines: an immunological perspective. *Developmental*
576 *and comparative immunology* 23, 459-472. doi:10.1016/s0145-305x(99)00028-2.

577 Casals-Casas, C., Desvergne, B., 2011. Endocrine disruptors: from endocrine to metabolic
578 disruption. *Annual review of physiology* 73, 135-162. doi:10.1146/annurev-physiol-
579 012110-142200.

580 Chen, L., et al., 2019a. TiO₂ nanoparticles and BPA are combined to impair the development
581 of offspring zebrafish after parental coexposure. *Chemosphere* 217, 732-741.
582 doi:10.1016/j.chemosphere.2018.11.052.

583 Chen, M., et al., 2019b. Changes in hepatic metabolic profile during the evolution of STZ-
584 induced diabetic rats via an (1)H NMR-based metabonomic investigation. *Bioscience*
585 *reports* 39. doi:10.1042/BSR20181379.

586 Chen, R., et al., 2016. DNA damage-inducible transcript 4 (DDIT4) mediates
587 methamphetamine-induced autophagy and apoptosis through mTOR signaling pathway
588 in cardiomyocytes. *Toxicology and applied pharmacology* 295, 1-11.
589 doi:10.1016/j.taap.2016.01.017.

590 Chevalier, N., Fenichel, P., 2015. Bisphenol A: Targeting metabolic tissues. *Reviews in*
591 *endocrine & metabolic disorders* 16, 299-309. doi:10.1007/s11154-016-9333-8.

592 Chevalier, N., Fenichel, P., 2016. [Endocrine disruptors: A missing link in the pandemy of
593 type 2 diabetes and obesity?]. *Presse medicale* 45, 88-97.
594 doi:10.1016/j.lpm.2015.08.008.

595 Coimbra, A.M., et al., 2015. Chronic effects of clofibric acid in zebrafish (*Danio rerio*): a
596 multigenerational study. *Aquatic toxicology* 160, 76-86.
597 doi:10.1016/j.aquatox.2015.01.013.

598 Crump, D., et al., 2002. Exposure to the herbicide acetochlor alters thyroid hormone-
599 dependent gene expression and metamorphosis in *Xenopus Laevis*. *Environmental*
600 *health perspectives* 110, 1199-1205. doi:10.1289/ehp.021101199.

601 DeCourten, B.M., et al., 2019. Direct and indirect parental exposure to endocrine disruptors
602 and elevated temperature influences gene expression across generations in a
603 euryhaline model fish. *PeerJ* 7, e6156. doi:10.7717/peerj.6156.

604 Duarte-Guterman, P., et al., 2014. Mechanisms of crosstalk between endocrine systems:
605 regulation of sex steroid hormone synthesis and action by thyroid hormones. *General
606 and comparative endocrinology* 203, 69-85. doi:10.1016/j.ygcen.2014.03.015.

607 Faitova, J., et al., 2006. Endoplasmic reticulum stress and apoptosis. *Cellular & molecular
608 biology letters* 11, 488-505. doi:10.2478/s11658-006-0040-4.

609 Fedorenkova, A., et al., 2012. Ranking ecological risks of multiple chemical stressors on
610 amphibians. *Environmental toxicology and chemistry* 31, 1416-1421.
611 doi:10.1002/etc.1831.

612 Ferrannini, E., et al., 1990. Influence of long-term diabetes on liver glycogen metabolism in
613 the rat. *Metabolism: clinical and experimental* 39, 1082-1088. doi:10.1016/0026-
614 0495(90)90170-h.

615 Fitzpatrick, L.C., 1976. Life history patterns of storage and utilization of lipids for energy in
616 amphibians. *American zoologist* 16, 725-732

617 Foroughi, M., et al., 2016. Relationship between non-alcoholic fatty liver disease and
618 inflammation in patients with non-alcoholic fatty liver. *Advanced biomedical research* 5,
619 28. doi:10.4103/2277-9175.176368.

620 Furfaro, A.L., et al., 2012. Impaired synthesis contributes to diabetes-induced decrease in
621 liver glutathione. *International journal of molecular medicine* 29, 899-905.
622 doi:10.3892/ijmm.2012.915.

623 Gomez-Mestre, I., et al., 2013. Mechanisms and consequences of developmental
624 acceleration in tadpoles responding to pond drying. *PloS one* 8, e84266.
625 doi:10.1371/journal.pone.0084266.

626 Gomez-Valades, A.G., et al., 2008. Pck1 gene silencing in the liver improves glycemia
627 control, insulin sensitivity, and dyslipidemia in db/db mice. *Diabetes* 57, 2199-2210.
628 doi:10.2337/db07-1087.

629 Halle, M., et al., 2007. Protein tyrosine phosphatases: emerging regulators of apoptosis. *Cell*
630 *Cycle* 6, 2773-2781. doi:10.4161/cc.6.22.4926.

631 Hammond, S.A., et al., 2015. Influence of temperature on thyroid hormone signaling and
632 endocrine disruptor action in *Rana* (*Lithobates*) *catesbeiana* tadpoles. *General and*
633 *comparative endocrinology* 219, 6-15. doi:10.1016/j.ygcen.2014.12.001.

634 Hayes, T.B., et al., 2011. Demasculinization and feminization of male gonads by atrazine:
635 consistent effects across vertebrate classes. *The Journal of steroid biochemistry and*
636 *molecular biology* 127, 64-73. doi:10.1016/j.jsbmb.2011.03.015.

637 Hayes, T.B., et al., 2006. Pesticide mixtures, endocrine disruption, and amphibian declines:
638 are we underestimating the impact? *Environmental health perspectives* 114 Suppl 1, 40-
639 50.

640 Hayes, T.B., et al., 2002. Hermaphroditic, demasculinized frogs after exposure to the
641 herbicide atrazine at low ecologically relevant doses. *Proceedings of the National*
642 *Academy of Sciences of the United States of America* 99, 5476-5480.
643 doi:10.1073/pnas.082121499.

644 Hayes, T.B., et al., 2010a. The cause of global amphibian declines: a developmental
645 endocrinologist's perspective. *Journal of Experimental Biology* 213, 921-933.
646 doi:10.1242/jeb.040865.

647 Hayes, T.B., et al., 2010b. Atrazine induces complete feminization and chemical castration in
648 male African clawed frogs (*Xenopus laevis*). *Proceedings of the National Academy of*
649 *Sciences of the United States of America* 107, 4612-4617.
650 doi:10.1073/pnas.0909519107.

651 Health Canada, 2016. Guidelines for Canadian Drinking Water Quality: Guideline Technical
652 Document — Benzo[a]pyrene. Water and Air Quality Bureau, Healthy Environments and
653 Consumer Safety Branch, Health Canada, Ottawa, Ontario.

654 Hedayatirad, M., et al., 2020. Transgenerational disrupting impacts of atrazine in zebrafish:
655 Beneficial effects of dietary spirulina. *Comparative biochemistry and physiology.*
656 *Toxicology & pharmacology : CBP* 230, 108685. doi:10.1016/j.cbpc.2019.108685.

657 Helbing, C.C., 2012. The metamorphosis of amphibian toxicogenomics. *Frontiers in genetics*
658 3, 37. doi:10.3389/fgene.2012.00037.

659 IUCN, 2019. The IUCN Red List of Threatened Species. Version 2019-3.

660 Kaloyianni, M., Freedland, R.A., 1990. Contribution of several amino acids and lactate to
661 gluconeogenesis in hepatocytes isolated from rats fed various diets. *The Journal of*
662 *nutrition* 120, 116-122. doi:10.1093/jn/120.1.116.

663 Kiesecker, J.M., 2002. Synergism between trematode infection and pesticide exposure: a link
664 to amphibian limb deformities in nature? *Proceedings of the National Academy of*
665 *Sciences of the United States of America* 99, 9900-9904. doi:10.1073/pnas.152098899.

666 Langlois, V.S., et al., 2010. Low levels of the herbicide atrazine alter sex ratios and reduce
667 metamorphic success in *Rana pipiens* tadpoles raised in outdoor mesocosms.
668 *Environmental health perspectives* 118, 552-557. doi:10.1289/ehp.0901418.

669 Lee, J.H., et al., 2010. A novel role for the dioxin receptor in fatty acid metabolism and
670 hepatic steatosis. *Gastroenterology* 139, 653-663. doi:10.1053/j.gastro.2010.03.033.

671 Lee, M.K., Blumberg, B., 2019. Transgenerational effects of obesogens. *Basic & clinical*
672 *pharmacology & toxicology* 125 Suppl 3, 44-57. doi:10.1111/bcpt.13214.

673 Major, K.M., et al., 2020. Early life exposure to environmentally relevant levels of endocrine
674 disruptors drive multigenerational and transgenerational epigenetic changes in a fish
675 model. *Frontiers in Marine Science* 7. doi:10.3389/fmars.2020.00471.

676 Moyer, B.J., et al., 2016. Inhibition of the aryl hydrocarbon receptor prevents Western diet-
677 induced obesity. Model for AHR activation by kynurenine via oxidized-LDL, TLR2/4,
678 TGFbeta, and IDO1. *Toxicology and applied pharmacology* 300, 13-24.
679 doi:10.1016/j.taap.2016.03.011.

680 Musso, G., et al., 2013. Cholesterol metabolism and the pathogenesis of non-alcoholic
681 steatohepatitis. *Progress in lipid research* 52, 175-191.
682 doi:10.1016/j.plipres.2012.11.002.

683 Naik, A., et al., 2013. Genomic aspects of NAFLD pathogenesis. *Genomics* 102, 84-95.
684 doi:10.1016/j.ygeno.2013.03.007.

685 Olivares-Rubio, H.F., et al., 2015. Relationship between biomarkers and endocrine-disrupting
686 compounds in wild *Girardinichthys viviparus* from two lakes with different degrees of
687 pollution. *Ecotoxicology* 24, 664-685. doi:10.1007/s10646-014-1414-4.

688 Opitz, R., et al., 2005. Description and initial evaluation of a *Xenopus* metamorphosis assay
689 for detection of thyroid system-disrupting activities of environmental compounds.
690 *Environmental toxicology and chemistry* 24, 653-664.

691 Orton, F., Tyler, C.R., 2015. Do hormone-modulating chemicals impact on reproduction and
692 development of wild amphibians? *Biological reviews of the Cambridge Philosophical*
693 *Society* 90, 1100-1117. doi:10.1111/brv.12147.

694 Otda, T., et al., 2013. Proteasome dysfunction mediates obesity-induced endoplasmic
695 reticulum stress and insulin resistance in the liver. *Diabetes* 62, 811-824.
696 doi:10.2337/db11-1652.

697 Pei, L., et al., 2006. NR4A orphan nuclear receptors are transcriptional regulators of hepatic
698 glucose metabolism. *Nature medicine* 12, 1048-1055. doi:10.1038/nm1471.

699 Perera, B.P.U., et al., 2020. The role of environmental exposures and the epigenome in
700 health and disease. *Environmental and molecular mutagenesis* 61, 176-192.
701 doi:10.1002/em.22311.

702 Petersen, G., et al., 2007. Study on enhancing the Endocrine Disrupter priority list with a
703 focus on low production volume chemicals (European Commission). Available from:
704 http://ec.europa.eu/environment/chemicals/endocrine/pdf/final_report_2007.pdf.

705 Regnault, C., et al., 2018. Unexpected metabolic disorders induced by endocrine disruptors
706 in *Xenopus tropicalis* provide new lead for understanding amphibian decline.
707 *Proceedings of the National Academy of Sciences of the United States of America* 115,
708 E4416-E4425. doi:10.1073/pnas.1721267115.

709 Regnault, C., et al., 2016. Metabolic and immune impairments induced by the endocrine
710 disruptors benzo[a]pyrene and triclosan in *Xenopus tropicalis*. *Chemosphere* 155, 519-
711 527. doi:10.1016/j.chemosphere.2016.04.047.

712 Regnault, C., et al., 2014. Impaired liver function in *Xenopus tropicalis* exposed to
713 benzo[a]pyrene: transcriptomic and metabolic evidence. *BMC genomics* 15, 666.
714 doi:10.1186/1471-2164-15-666.

715 Reid, B.N., et al., 2008. Hepatic overexpression of hormone-sensitive lipase and adipose
716 triglyceride lipase promotes fatty acid oxidation, stimulates direct release of free fatty
717 acids, and ameliorates steatosis. *The Journal of biological chemistry* 283, 13087-13099.
718 doi:10.1074/jbc.M800533200.

719 Robert, J., et al., 2018. Water Contaminants Associated With Unconventional Oil and Gas
720 Extraction Cause Immunotoxicity to Amphibian Tadpoles. *Toxicological sciences : an*
721 *official journal of the Society of Toxicology* 166, 39-50. doi:10.1093/toxsci/kfy179.

722 Robert, J., et al., 2019. Developmental exposure to chemicals associated with
723 unconventional oil and gas extraction alters immune homeostasis and viral immunity of
724 the amphibian *Xenopus*. *The Science of the total environment* 671, 644-654.
725 doi:10.1016/j.scitotenv.2019.03.395.

726 Safholm, M., et al., 2014. Risks of hormonally active pharmaceuticals to amphibians: a
727 growing concern regarding progestagens. *Philosophical transactions of the Royal*
728 *Society of London. Series B, Biological sciences* 369. doi:10.1098/rstb.2013.0577.

729 Showell, C., Conlon, F.L., 2009. Natural mating and tadpole husbandry in the western
730 clawed frog *Xenopus tropicalis*. *Cold Spring Harbor protocols* 2009, pdb prot5292.
731 doi:10.1101/pdb.prot5292.

732 Skinner, M.K., 2014. Endocrine disruptor induction of epigenetic transgenerational
733 inheritance of disease. *Molecular and cellular endocrinology* 398, 4-12.
734 doi:10.1016/j.mce.2014.07.019.

735 Smith, D., 1987. Adult recruitment in chorus frogs: effects of size and date at metamorphosis.
736 *Ecology* 68, 344-350.

737 Swain, P., Nayak, S.K., 2009. Role of maternally derived immunity in fish. *Fish & shellfish*
738 *immunology* 27, 89-99. doi:10.1016/j.fsi.2009.04.008.

739 Takahashi, Y., Fukusato, T., 2014. Histopathology of nonalcoholic fatty liver
740 disease/nonalcoholic steatohepatitis. *World journal of gastroenterology* 20, 15539-
741 15548. doi:10.3748/wjg.v20.i42.15539.

742 Tanos, R., et al., 2012. Aryl hydrocarbon receptor regulates the cholesterol biosynthetic
743 pathway in a dioxin response element-independent manner. *Hepatology* 55, 1994-2004.
744 doi:10.1002/hep.25571.

745 Trapido, M., Ingeborg, V., 1996. On polynuclear aromatic hydrocarbons contamination levels
746 in the ecosystem of Lake Peipsi in the 1970s-1980s. . *Hydrobiologia* 338 185-190.

747 Urbatzka, R., et al., 2007. Endocrine disruptors with (anti)estrogenic and (anti)androgenic
748 modes of action affecting reproductive biology of *Xenopus laevis*: I. Effects on sex
749 steroid levels and biomarker expression. *Comparative biochemistry and physiology.*
750 *Toxicology & pharmacology : CBP* 144, 310-318. doi:10.1016/j.cbpc.2006.10.008.

751 Usal, M., et al., 2019. Concomitant exposure to benzo[a]pyrene and triclosan at
752 environmentally relevant concentrations induces metabolic syndrome with
753 multigenerational consequences in *Silurana (Xenopus) tropicalis*. *The Science of the*
754 *total environment* 689, 149-159. doi:10.1016/j.scitotenv.2019.06.386.

755 Viluksela, M., Pohjanvirta, R., 2019. Multigenerational and Transgenerational Effects of
756 Dioxins. *International journal of molecular sciences* 20. doi:10.3390/ijms20122947.

757 Vincenz, L., et al., 2013. Endoplasmic reticulum stress and the unfolded protein response:
758 targeting the Achilles heel of multiple myeloma. *Molecular cancer therapeutics* 12, 831-
759 843. doi:10.1158/1535-7163.MCT-12-0782.

760 WHO, 2003. Polynuclear aromatic hydrocarbons in Drinking-water. Background document
761 for development of WHO., *Guidelines for drinking-water quality*, 2nd ed, Addendum to
762 Vol. 2. Health criteria and other supporting information. (Eds) World Health Organization,
763 Geneva.

764 WHO, 2012. State of the science of endocrine disrupting chemicals in: Bergman, A.,
765 Heindelerrold, J.J., Jobling, S., Kidd, K.A., Zoeller, T. (Eds.). World Health Organization.,
766 Geneva.

767 Wise, S., et al., 1997. Effects of hepatic glycogen content on hepatic insulin action in
768 humans: alteration in the relative contributions of glycogenolysis and gluconeogenesis to
769 endogenous glucose production. *The Journal of clinical endocrinology and metabolism*
770 82, 1828-1833. doi:10.1210/jcem.82.6.3971.

771 Wright, K., 2003. Cholesterol, corneal lipidosis, and xanthomatosis in amphibians. *The*
772 *veterinary clinics of North America. Exotic animal practice* 6, 155-167.
773 doi:10.1016/s1094-9194(02)00022-1.

774 Xie, X., et al., 2019. Underlying mechanisms of reproductive toxicity caused by
775 multigenerational exposure of 2, bromo-4, 6-dinitroaniline (BDNA) to Zebrafish (*Danio*
776 *rerio*) at environmental relevant levels. *Aquatic toxicology* 216, 105285.
777 doi:10.1016/j.aquatox.2019.105285.

778 Yamaguchi, M., Murata, T., 2013. Involvement of regucalcin in lipid metabolism and
779 diabetes. *Metabolism: clinical and experimental* 62, 1045-1051.
780 doi:10.1016/j.metabol.2013.01.023.

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784 **Fig. captions**

785 **Fig. 1. Multigenerational effects of BaP on F1 females.** **a.** Hepatosomatic index of F1-
786 control and F1-BaP females. ($n_{\text{Control}} = 6$, $n_{\text{BaP}} = 21$). **b.** Adiposomatic index of control and
787 F1-BaP females. ($n_{\text{Control}} = 6$, $n_{\text{BaP}} = 21$). **c.** Hepatic lipid content of F1-control and F1-BaP
788 females. The images reveal the lipid droplets (red staining, Oil Red O; Scale bars, 75 μm).
789 The histogram represents the proportion of stained area in the livers. ($n_{\text{Control}} = 6$, $n_{\text{BaP}} = 10$).
790 **d.** Hematoxylin and erythrosin staining of liver sections from F1-control females (Scale bars,
791 120 μm). **e.** Hematoxylin and erythrosin staining of liver sections from F1-BaP females (Scale
792 bars, 120 μm). The asterisks indicate leukocyte infiltrates and the arrow indicates a dilated
793 blood vessel. **f.** Hematoxylin and erythrosin staining of liver sections from F1-control females
794 (Scale bars, 60 μm). **g.** Hematoxylin and erythrosin staining of liver sections from F1-BaP
795 females (Scale bars, 60 μm). The asterisk indicates leukocyte infiltrate. ($n_{\text{Control}} = 6$, $n_{\text{BaP}} =$
796 10). **h.** Fasting glycaemia of F1-control and F1-BaP females ($n_{\text{Control}} = 6$, $n_{\text{BaP}} = 21$). **i.**
797 Fasting plasma insulin concentrations of F1-control and F1-BaP females ($n_{\text{Control}} = 4$, $n_{\text{BaP}} =$
798 8). **j.** Muscle glycogen content of F1-control and F1-BaP females ($n_{\text{Control}} = 6$, $n_{\text{BaP}} = 10$). **k.**
799 Liver glycogen content of F1-control and F1-BaP females ($n_{\text{Control}} = 6$, $n_{\text{BaP}} = 10$). Statistical
800 analyses used Wilcoxon's test. Statistical analyses used Wilcoxon's test. The asterisks
801 indicate a significant difference from the control: $**P < 0.01$. **l.** Circos plot representing the
802 overlap between the liver transcriptome of F1-BaP females and metabolic pathways. The
803 genes considered are those involved in metabolic disorders, immune system pathway and
804 histopathological consequences. Dysregulated genes and pathways are indicated outside
805 the circle. The color scale indicates transcription ratios (\log_2 fold change) relative to the F1-
806 control female. ($n_{\text{Control}} = 5$, $n_{\text{BaP}} = 6$).

807

808 **Fig. 2. Transgenerational developmental and metabolic effects of BaP on F2 females.**

809 **a.** Development curves of F2 individuals from hatching to metamorphosis. The distributions
810 of F2 individual development times were compared between the F2-control population and

811 F2-BaP population using a Kaplan-Meyer test to evaluate global curve difference ($n_{\text{Control}} =$
812 216, $n_{\text{BaP}} = 134$). The asterisks indicate a significant difference from the control: $***$, $p <$
813 0.001.. **b.** Development curves of F2 juvenile females from hatching to sexual maturity. The
814 distributions of F2 female development times were compared between F2-control
815 populations and F2-BaP population separately, using a Kaplan-Meyer test to evaluate global
816 curve difference ($n_{\text{Control}} = 87$, $n_{\text{BaP}} = 44$). The asterisks indicate a significant difference from
817 the control: $***$, $p < 0.001$. **c.** Hepatosomatic index of F2-control and F2-BaP females. (n_{Control}
818 $= 16$, $n_{\text{BaP}} = 16$). **d.** Adiposomatic index of F2-control and F2-BaP females. ($n_{\text{Control}} = 16$, n_{BaP}
819 $= 16$). **e.** Hematoxylin and erythrosin staining of liver sections from F2-control females (Scale
820 bars, 75 μm). **f.** Hematoxylin and erythrosin staining of liver sections from F2-BaP females
821 (Scale bars, 75 μm). The asterisks indicate leukocyte infiltrates and the arrow indicates a
822 dilated blood vessel. **g.** Hematoxylin and erythrosin staining of liver sections from F2-control
823 females (Scale bars, 40 μm). **h.** Hematoxylin and erythrosin staining of liver sections from
824 F2-BaP females (Scale bars, 40 μm). The asterisk indicates leukocyte infiltrate ($n_{\text{Control}} = 10$,
825 $n_{\text{BaP}} = 10$). **i.** Fasting glycaemia of F2-control and F2-BaP females ($n_{\text{Control}} = 36$, $n_{\text{BaP}} = 22$). **j.**
826 Fasting plasma insulin concentrations of F2-control and F2-BaP females ($n_{\text{Control}} = 10$, $n_{\text{BaP}} =$
827 10). **k.** Glucose tolerance test ($n_{\text{Control}} = 21$, $n_{\text{BaP}} = 12$). **l.** Muscle glycogen content of F2-
828 control and F2-BaP females ($n_{\text{Control}} = 16$, $n_{\text{BaP}} = 13$). **m.** Insulin resistance test ($n_{\text{Control}} = 10$,
829 $n_{\text{BaP}} = 8$). **n.** Plasma insulin concentrations 30 minutes after glucose injection ($n_{\text{Control}} = 6$, n_{BaP}
830 $= 6$). **o.** Percentage of F2 females with pancreatic inflammation 30 minutes after glucose
831 injection ($n_{\text{Control}} = 6$, $n_{\text{BaP}} = 6$). Images show insulin immunostaining of pancreas from F2-
832 control and F2-BaP females. Asterisks indicate leukocyte infiltrates. Statistical analyses were
833 performed using Wilcoxon's test (c-d, i-n) and a χ^2 test (o). The asterisks indicate a
834 significant difference from the control: $* P < 0.05$, $**P < 0.01$.

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838 **Fig. 3. Liver transcriptomic and metabolomic signatures of F2-BaP females.** **a.** Circos
839 plot representing the overlap between liver transcriptome of F2-BaP females and metabolic
840 pathways. The genes considered are those involved in metabolic disorders, immune system
841 pathway and histopathological consequences. Dysregulated genes and pathways are
842 indicated outside the circle. The color scale indicates transcription ratios (log₂ fold change)
843 relative to F2-control females (n_{Control} = 5, n_{BaP} = 5). **b.** Percent change in discriminant
844 metabolites between liver from F2-control and F2-BaP females (n_{Control} = 10, n_{BaP} = 10).
845 Statistical analyses were performed using Wilcoxon's test with multiple testing correction.
846 The asterisks indicate a significant difference from the control: * $P < 0.05$, ** $P < 0.01$, *** $P <$
847 0.001 . *Un. Fatty acids*: unsaturated fatty acids.

848

849 **Fig. 4. F2-BaP reproductive capacities.** **a.** Gonadosomatic index of F2-control and F2-BaP
850 females (n_{Control} = 16, n_{BaP} = 16). **b.** Percent change in discriminant metabolites between
851 eggs from the control and F2-BaP females (n_{Control} = 12, n_{BaP} = 12). *MU. Fatty acids*: Mono-
852 unsaturated fatty acids. **c.** Percentage of females having laid eggs (n_{Control} = 5, n_{BaP} = 5). **d.**
853 Percentage of successful amplexus (n_{Control} = 5, n_{BaP} = 5). **e.** Number of hatched eggs per
854 mating pairs (n_{Control} = 5, n_{BaP} = 5).

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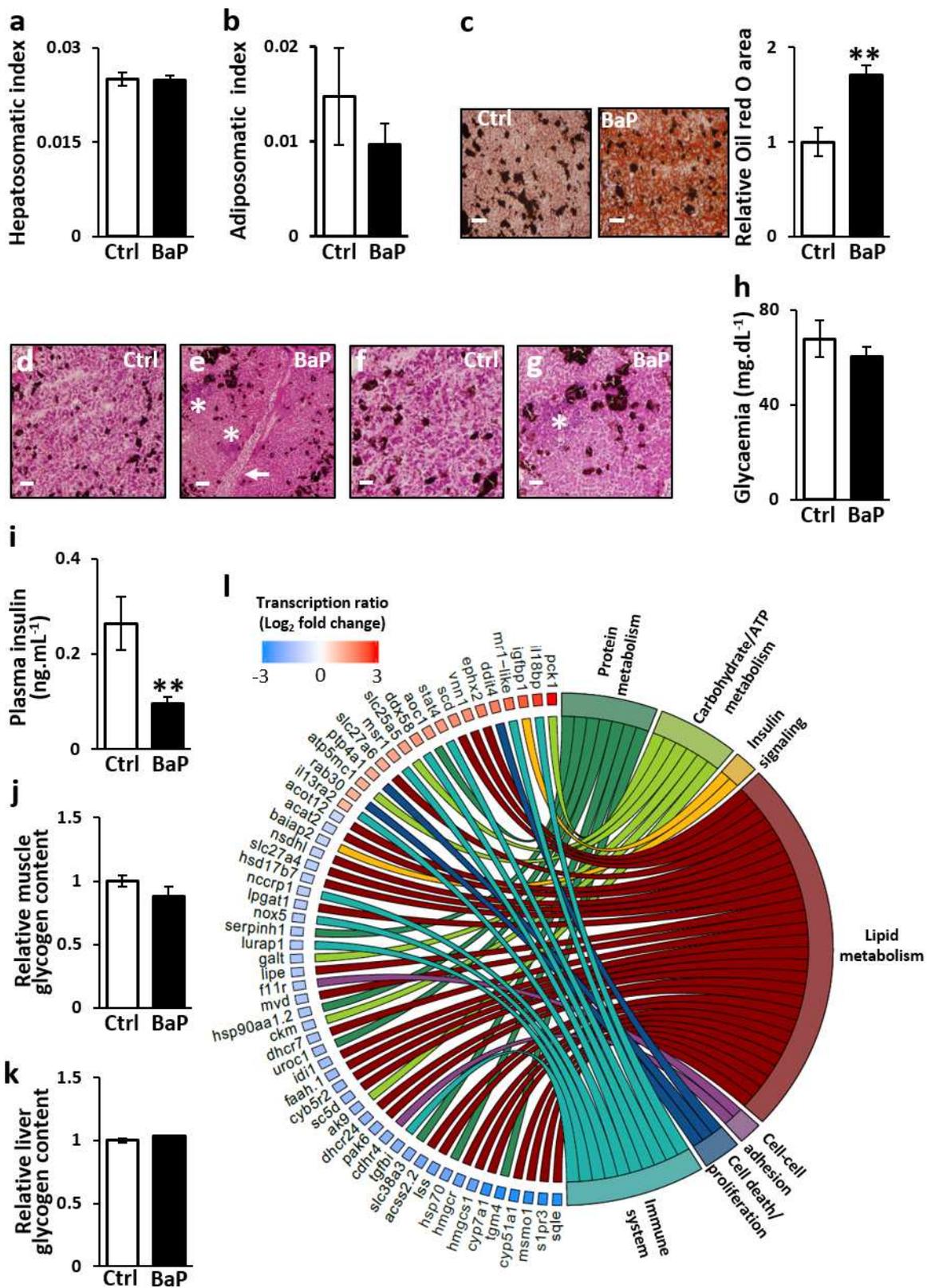
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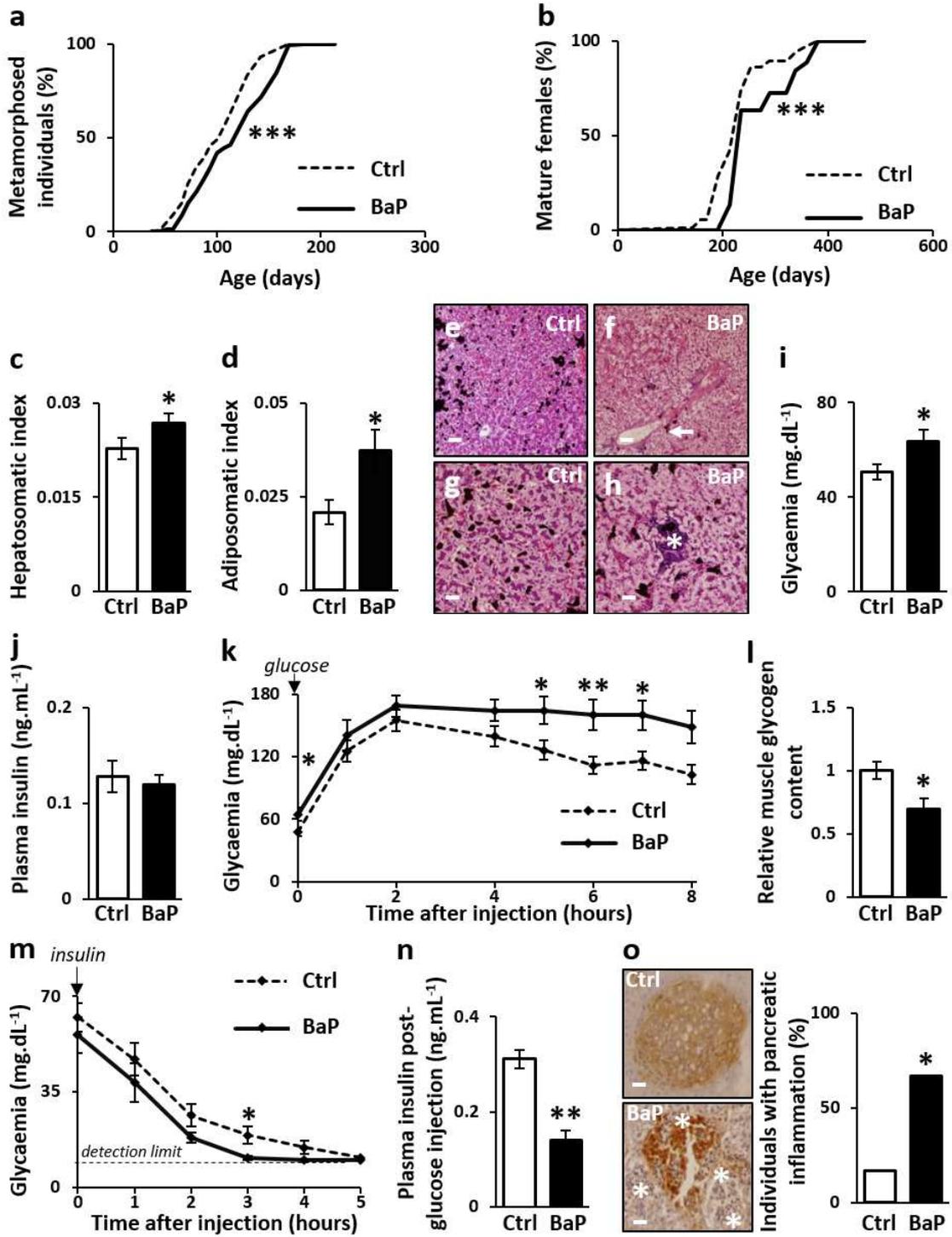
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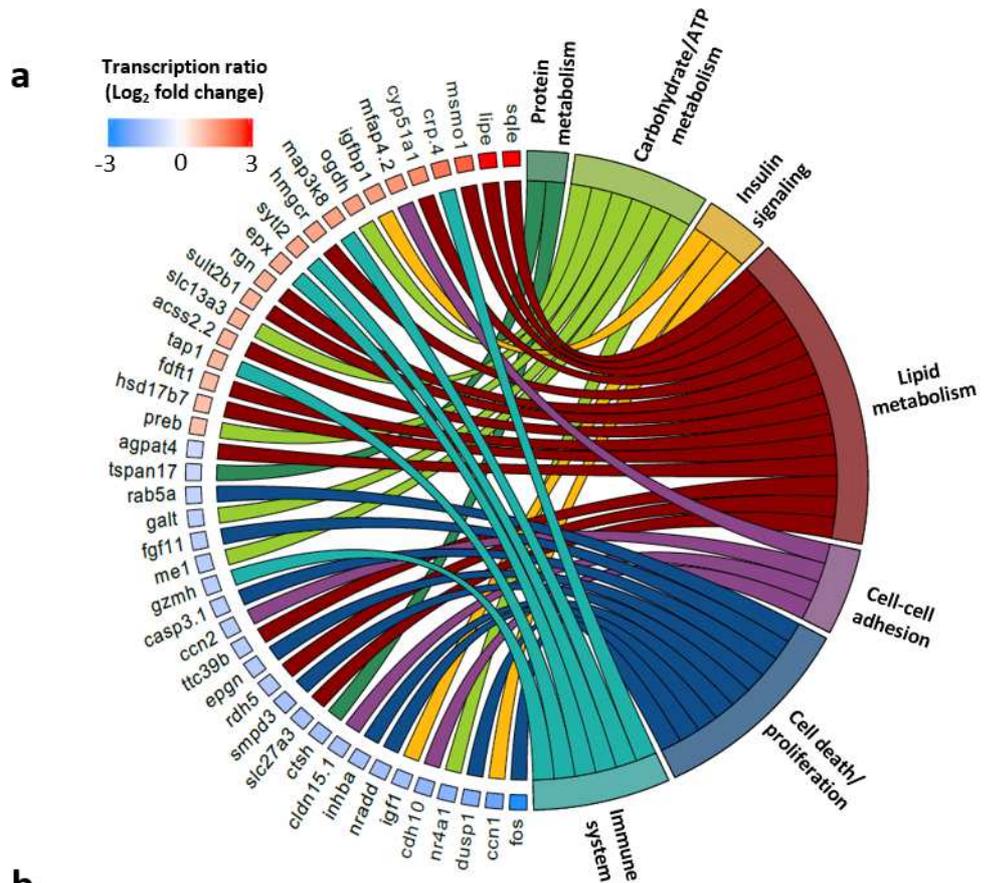
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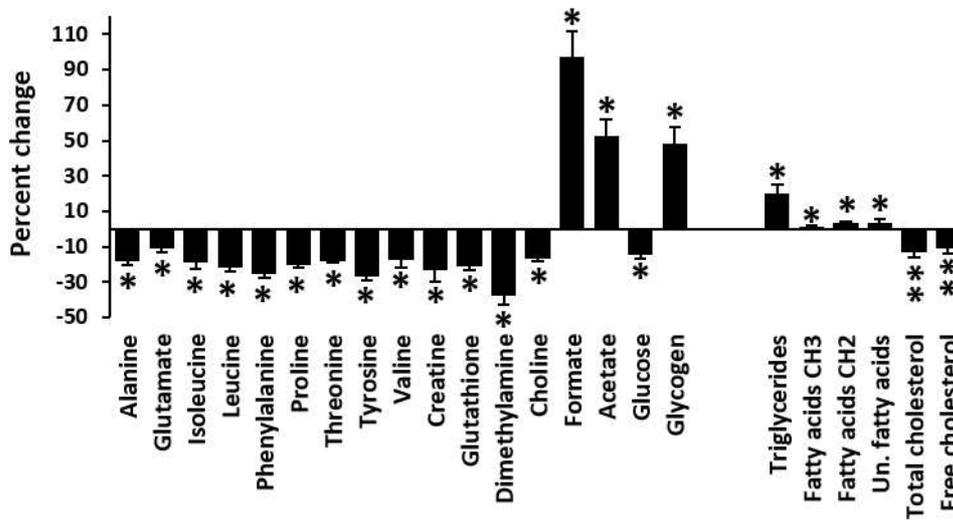


871 **Fig.3**

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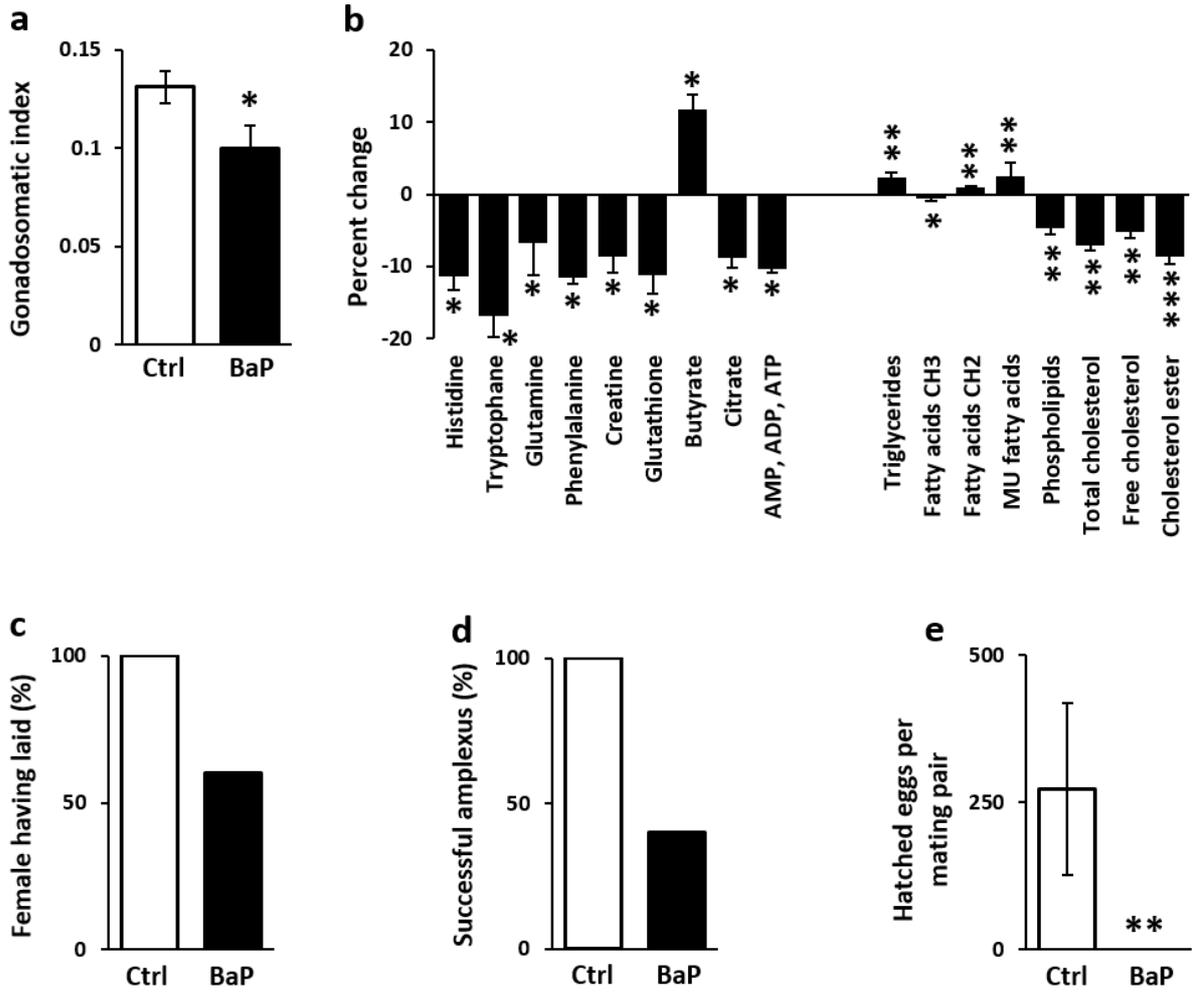
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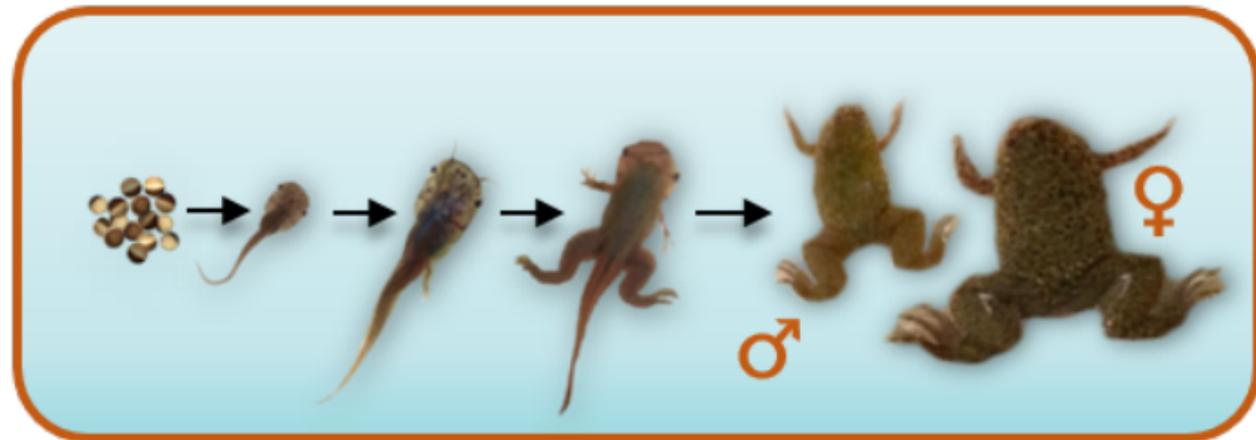
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F0 generation (exposed to BaP)



F2 (unexposed generation)

