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1 Epoxiconazole alters the histology and transcriptome of mouse liver in a transgenerational
2 pattern.

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24 **Abstract**

25 Background: The use of phytosanitary products is always associated with their safety concern
26 on the environment and on health. The associated adverse effects are very broad and
27 substance-dependent. Among these substances, epoxiconazole (EPOX) is one of the most
28 widely used fungicides, especially in beet crops. Although its use is questionable or even
29 prohibited in the European Union, it is still widely used and its consequences
30 (transgenerational effects) on future generations is unknown.

31 Objectives: We aimed to investigate the hepatic effects of epoxiconazole in the descendants of
32 perinatally exposed low-dose C57Bl/6J mice, focusing on liver histological and
33 transcriptomic analyses.

34 Methods: From day 0 of gestation up to day 21 postnatal, only pregnant F0 C57BL6/J mice
35 were exposed to EPOX (1.75 µg/kg bw/day). F1 males and females were mated to obtain the
36 F2 generation and similarly, F2 mice were crossed to obtain F3. Histological and
37 transcriptomic analyses of the liver were performed. Gene set enrichment analysis was
38 realized to determine an *a priori* defined set of genes with significantly altered mRNA
39 expression. Plasma parameters were also measured.

40 Results and conclusions: Perinatal exposure to EPOX induces transgenerational effects with
41 phenotypic, histological and transcriptomic changes in the liver. These changes are highly
42 dependent on the sex and generation of the animal. All these modifications lead to an
43 alteration of the hepatic metabolism resulting in a difference in the size of the hepatocytes.
44 Beyond these specific mechanisms, EPOX also seems to have a more general impact on
45 hepatic metabolism *via* the circadian rhythm.

46 Keywords: transgenerational effects, epoxiconazole, low dose and perinatal exposure,
47 hepatotoxicity, Endocrine Disruptor

48

49 **1. Introduction**

50 Exposures to endocrine disrupting pesticides are likely to contribute substantially to
51 diseases and physiological dysfunctions throughout life (Trasande et al., 2015). Adverse
52 effects of endocrine disruptors affect exposed individuals, but can also alter the health of their
53 descendants as well as subsequent generations even if they have not directly been exposed.
54 Most of these so-called transgenerational effects are most often observed following perinatal
55 exposure (Diamanti-Kandarakis et al., 2009; Gore, 2015). These responses often occur with a
56 specific gender effect in the offspring due to the existence of different environmental
57 adaptation mechanisms between males and females (Curley et al., 2011; Dunn et al., 2011;
58 Sugathan and Waxman, 2013). They obviously depend on the nature, the window and the
59 duration of the exposure, and the dose. Among the multitude of compounds used in
60 agriculture, which are very controversial, epoxiconazole (EPOX) has been the subject of
61 many criticisms for its potential effects on human health. EPOX ((2RS, 3SR) -1- [3- (2-
62 chlorophenyl) -2,3-epoxy-2- (4-fluorophenyl) propyl] -1H-1,2,4-triazole, is an active
63 substance of plant protection products belonging to the triazole class and used in the control
64 of various fungal diseases in cereals, rice, sugar beets, bananas, coffee and soybeans. EPOX
65 like all azole fungicides inhibits ergosterol demethylase (CYP51), an essential enzyme for
66 fungal membrane synthesis, resulting in membrane disruption. The tolerable daily intake
67 (TDI) defined by EFSA is 8 µg/kg bw/d (based on a two-generation reproduction study in the
68 rat) and the acute reference dose (ARf) is 0.023 mg/kg bw in the same species. Regarding oral
69 exposure, EFSA estimates it to be less than 17% of the TDI, with sugar beet root being the
70 main contributor to the dietary exposure. For consumers, assessments of the theoretical
71 maximum daily intake (MTDD) at international and national levels showed that chronic
72 exposure is less than 20% (1.6 µg/kg bw/d) of the epoxiconazole TDI. Several studies have
73 evaluated the harmful effects of EPOX in mammals, but almost all of the studies have been

74 carried out with very high doses (> 10 mg /kg bw /d) which is not representative of exposure
75 levels in humans. These studies showed that EPOX could induce reprotoxic and hepatotoxic
76 effects (with a potential development of hepatocarcinoma (DAR, 2006)). EPOX is a
77 carcinogenic, mutagenic and reprotoxic (CMR) substance classified in substances C2 and
78 R1B (European Regulation 1272/2008 modified by UE944/2013, annex II). EPOX also has
79 endocrine disrupting activity as it has been described *in vitro* as a weak estrogenic compound
80 and an anti-androgen (Vinggaard et al., 1999). EPOX is also able to inhibit the production of
81 testosterone and estradiol in H295R cells (steroid synthesis test) (Wuttke et al., 2001). Indeed,
82 EPOX is an inhibitor of CYP 450 enzyme, such as aromatase involved in the synthesis of
83 estrogen and lanosterol 14 α -demethylase (or CYP51A1), responsible for an essential step in
84 the biosynthesis of sterols and then of steroids (Lepesheva et al., 2008). However, high doses
85 of EPOX appear to be hepatotoxic and impair body weight gain in rodent studies (Goetz et al.,
86 2007). In addition, there are no studies regarding the biological effect of low dose EPOX
87 and/or at environmental doses. Another important concern regarding endocrine disruptors is
88 their potential effects in a generational or transgenerational model.

89 Here, we analyzed hepatotoxicity after perinatal exposure of the F1 generation to
90 EPOX at 1.75 μ g/kg bw/day. We also investigated these disturbances on three generations
91 (F1, F2 and F3) of male and female C57BL6 / J mice. Analyses were focused on the liver
92 histology and transcriptome profile.

93

94 **2. Methods**

95 2.1. Animals and materials

96 Pregnant C57Bl/6J mice were provided by Charles Rivers. Epoxiconazole was
97 purchased from Sigma-Aldrich CAS no. 133855-98-8. Diet (4RF21; Mucedola 4RF2) is
98 certified as estrogen free (phytoestrogen concentration is lower than 4 ppb) and accurately

99 tested for the detection of estrogenic activities. Cholesterol FS™, Triglyceride FS™ and No
100 Esterified Fatty Acids (NEFA) FS™ kits were obtained from DiaSys. Blood glucose test
101 strips and glucometers (Accu-Check® Aviva, Roche Diagnostics) were provided from the
102 pharmacy of the University (Dijon, France).

103

104 2.2. Experimental design

105 The mice had free access to food and water with a 12-hour light-dark cycle and a
106 controlled room temperature of 22°C. Thirty-three F0 pregnant C57Bl/6J mice were
107 individually housed. F0 Mice were exposed to epoxiconazole from the first day of gestation
108 up to post-natal day 21 of F1 offspring

109 After mating, gestation day 0 (GD0) was determined in the morning by observation of
110 the vaginal plug. Mice of the F0 generation (16 females) were exposed to GD0 epoxiconazole
111 and exposure continued in F1 offspring until postnatal day 21 (JPN21). The epoxiconazole
112 was resuspended in ethanol and diluted in drinking water to 8.5 ng/ml and the final ethanol
113 concentration was 0.1%. The drinking water of the control group (17 females) contained only
114 0.1% ethanol. The average weekly exposure to epoxiconazole was determined by measuring
115 the mouse water consumption (equal to the difference in the weight of the water bottles over
116 one week). Thus, the average epoxiconazole intake was 1.75 (\pm SD 0.14) μ g/kg bw/d.

117 Ten week-old male and female mice were crossed to generate the mice of the F2 and
118 F3 generations. The mated mice came from different litters. At weaning, a maximum of six
119 mice were housed per cage, ensuring that the litters were mixed randomly after weaning to
120 minimize a possible litter effect. Based on these conditions and for each generation, 12 male
121 and female mice were analyzed.

122 At 23 weeks, 4h-fasting mice were euthanized. Before euthanasia, body weight, fat
123 and lean masses were measured. Then, the mice were anesthetized with isoflurane, the blood

124 was taken by cardiac puncture and the mice were euthanized by cervical dislocation. The liver
125 was drained of blood with physiological NaCl *via* the portal vein and weighed. A piece of the
126 right lateral hepatic lobe was fixed in 4% paraformaldehyde and embedded in paraffin.
127 Experimental protocol (Accreditation Number: #11422) was approved by the University of
128 Burgundy's ethic committee and accredited by the ministry. Animal experiments have been
129 carried out in accordance with EU Directive 2010/63/EU for animal experiments.

130

131 2.3. Body weight monitoring and corporal composition measurement

132 Mice were weighed weekly and before the euthanasia. Fat and lean masses were also
133 measured individually using a quantitative EchoMRI 500T™ (EchoMRI, Houston, USA).
134 Before each measurement, a calibration was performed according to the manufacturer's
135 instructions.

136 2.4. Food intake measurement

137 Food was weighed daily for four consecutive days. Daily food intake was defined as
138 the difference between the initial and remaining amounts of food after 24 h. Thus, the daily
139 consumption was calculated from the average of the data obtained for three consecutive days.
140 Particular attention was paid to spills and/or hoarding. Spilled food was weighed and
141 subtracted from the measurements if necessary.

142

143 2.5. Biochemical analysis

144 Blood was collected by cardiac puncture during the euthanasia and centrifuged at
145 2000g, 10 min and 4°C. The level of total plasma cholesterol, triglycerides, NEFA and insulin
146 were measured using respective kits and according to the manufacturer's instructions. Blood
147 glucose levels were determined from a tail blood sample collected before anaesthesia.

148

149 2.6. Histological analysis

150 Histology experiments were performed on the DimaCell platform (INSERM
151 UMR1231, Dijon). As described above, a piece of the right lateral hepatic lobe was
152 immediately fixed in 4% paraformaldehyde, then dehydrated and embedded in paraffin block.
153 Fixed liver sections (5 μ m) were stained with hematoxylin-eosin or sirius red (hepatic fibrosis
154 analysis). The sections were visualized by optical microscopy (in polarized light for sirius
155 red) ($\times 10$ for sirius red staining; Zeiss Axioscope A1 with a Jenoptic Gryphax camera and
156 $\times 40$ for hematoxylin-eosin staining; Axiovert 25; Zeiss). Images were analyzed with ImageJ
157 software. Four sections were performed for each mouse and three mice per group were
158 analyzed. Kupffer cells were counted over the entire area of four different areas of each
159 section. The hepatocyte size was measured on five clearly identifiable cells in four distinct
160 areas of each section. The quantification of sirius red labeling was carried out using an
161 algorithm (Visiolog software) on the entire surface of the section (excluding vascular
162 elements). For this part, three sections were analyzed for each mouse and three mice per
163 group were analyzed.

164 Transcriptomic analysis.

165 2.7 Transcriptomic analysis

166 A piece of the right lateral hepatic lobe was ground and homogenized (Sigma-Aldrich, Saint
167 Quentin Fallavier, France) in a Precellys TM 24 tissue homogenizer (Bertin technologies,
168 Montigny-le-Bretonneux, France) and using Tubes Lysing Matrix D TM (MP Biomedical,
169 Illkirch-Graffenstaden, France). For total RNA extraction, the liver sample was ground in Tri-
170 reagent TM and the classical phenol/chloroform method was performed. The quality of the
171 RNAs (RNA Integrity Number) and their quantification were carried out by capillary
172 electrophoresis (Agilent 2100 bioanalyzer, Les Ullis, France).

173 For the microarray assays, each sample consisted of a pool of RNA from the liver of
174 three separate mice from different litters. The constitution of the pools was based on the
175 quality of the nucleic acids in order to have three homogeneous samples. Equal amounts of
176 nucleic acid were used for each mouse. A total of 200 ng (66 ng RNA per mouse) was
177 required for microarray analysis. Thus, for each group (epoxiconazole and control), hepatic
178 RNAs from nine mice (three pools of three mice per microarray assay) were analyzed. The
179 composition of RNA pools was different as they depended on the respective nucleic acid
180 quality.

181 2.8 Microarray and bioinformatics analysis

182 Cartridges of Affymetrix Clariom S array hybridization were used to analyze the global gene
183 expression from isolated cRNA. The standard validated protocol were carried out at the
184 Genomics Platform of the Cochin Institute. The genes showing a significant deregulation
185 ($p \leq 0.05$) were converted to ENSEMBL ID with Biomart and mm10 version of the mouse
186 genome (<http://www.ensembl.org/biomart>). The .CEL type files were analyzed with the
187 Transcription Analysis Console 4.0 (TAC 4.0; Affymetrix, Applied Biosystems). Differential
188 expression in mRNA was shown as a pie chart and heat map. For downregulated or
189 upregulated genes, a functional gene set enrichment analysis (GSEA) was performed with
190 WebGestalt 2019 (WEB-based Gene Set AnaLysis Toolkit) (<http://www.webgestalt.org/>)
191 using KEGG pathway database.

192 2.9 Statistical analysis

193 All data were expressed as mean \pm standard deviation (SD) and considered statistically
194 significant different at $p < 0.05$ using Student's unpaired t-test. Gaussian distributions and equal
195 variances were previously verified with Graph Pad Prism® software. The Chi-square test was
196 performed to determine the difference in hepatocyte size between each group ($p < 0.05$).

197

198 **3. Results**

199 3.1. Multigenerational effects of epoxiconazole on physiological and plasma parameters

200 ***Body weight and corporal composition*** (Table 1): Exposure to epoxiconazole did not
201 induce any change in the body weight of the F1 and F2 mice regardless of sex. Only a
202 significant decrease ($p < 0.01$) in the weight of male F3 mice was observed (8% compared to
203 the control group). No changes were found in the body composition (fat and lean mass) of
204 these animals. In females, regards the body composition, only the fat mass of the F1 female
205 mice decreased in the EPOX ($9.3\% \text{ SD} \pm 2.4$; $p < 0.01$) groups compared to the control group.

206 ***Food intake and water consumption*** (Table 1): In males, decreases in food intake and
207 water consumption (normalized to body weight) were observed in F2 mice (5.3% compared to
208 the control group; $p < 0.01$) which were exacerbated in F3 mice (15.7% compared to the
209 control group; $p < 0.01$).

210 ***Serum lipids, glucose and insulin levels*** (Table 1): Whatever the sex and generation,
211 no differences were found in glucose and insulin plasma levels. In male mice, only the total
212 cholesterol plasma levels decreased in the F1 animals (30% compared to control group,
213 $p < 0.01$). No differences were observed for triglycerides and NEFA (Non-Esterified Fatty
214 Acids) plasma levels. In males of the F2 and F3 generation, there was no difference in these
215 blood parameters.

216 Female F1 mice exposed to epoxiconazole exhibited higher total cholesterol and
217 triglyceride plasma levels respectively 40 and 70% than in the control group. In females of the
218 F2 generation, effect on total cholesterol plasma level disappeared, but it persisted for
219 triglycerides with a 30% increase in the EPOX group compared to the control group. On the
220 other hand, an induction (170%) of the plasma level of NEFA was observed. No more
221 changes were observed in the F3 generation.

222

223 Table 1: Transgenerational study of epoxiconazole effects on physiological and plasma
 224 parameters in male (upper table) and female (lower table) mice.

	F1 male		F2 male		F3 male	
	CTRL	EPOX	CTRL	EPOX	CTRL	EPOX
Body Weight (g)	28.7 ± 2.1	28.6 ± 1.7	31.1 ± 2.84	30.4 ± 2.2	32.1 ± 1.87	29.5 ± 1.76
Fat mass (%)	10.7 ± 1.7	10.8 ± 1.1	13.7 ± 4.4	12.4 ± 2.4	13.6 ± 1.9	12.8 ± 3.0
Lean Mass (%)	76.9 ± 1.9	76.9 ± 2.2	74.1 ± 5.6	74.6 ± 3.6	74.4 ± 1.6	74.9 ± 3.2
Liver Weight (g)	1.21 ± 0.14	1.19 ± 0.15	1.32 ± 0.13	1.25 ± 0.12	1.28 ± 0.17	1.16 ± 0.09
Liver weight/Body weight (%)	4.24 ± 0.46	4.15 ± 0.43	4.27 ± 0.45	4.13 ± 0.37	4.01 ± 0.53	2.54 ± 0.19
Water intake (ml/kg bw/day)	122.9 ± 11.8	124.7 ± 9.8	120.1 ± 4.5	113.7 ± 3.1	113.1 ± 12.1	97.7 ± 12.0
Food Intake (kcal/kg bw/day)	485.9 ± 46.8	492.7 ± 38.7	474.7 ± 17.8	449.2 ± 12.2	447.1 ± 47.7	386.2 ± 47.6
Total Cholesterol (g/L)	0.63 ± 0.16	0.48 ± 0.15	0.57 ± 0.06	0.57 ± 0.08	0.54 ± 0.09	0.58 ± 0.10
Triglycerides (g/L)	0.42 ± 0.06	0.48 ± 0.17	0.66 ± 0.15	0.58 ± 0.12	0.39 ± 0.13	0.39 ± 0.08
NEFA (mmol/L)	0.44 ± 0.34	0.5 ± 0.39	0.49 ± 0.42	0.32 ± 0.29	0.27 ± 0.37	0.19 ± 0.23
Insuline (µg/ml)	0.94 ± 0.55	0.9 ± 0.67	1.23 ± 0.37	0.85 ± 0.69	0.67 ± 0.52	0.61 ± 0.20
Glucose (mg/dl)	164 ± 31.4	158.8 ± 37.7	147.3 ± 13.1	136.4 ± 22.63	151.5 ± 19.2	145.7 ± 22.2
Testosterone (pg/ml)	2 ± 1.2	1.4 ± 0.3	1.9 ± 0.3	2.4 ± 0.52	1.7 ± 0.41	1.9 ± 0.33

225

	F1 Female		F2 Female		F3 Female	
	CTRL	EPOX	CTRL	EPOX	CTRL	EPOX
Body Weight (g)	23.0 ± 1.4	22.6 ± 1.2	22.5 ± 1.0	22.5 ± 0.7	23.1 ± 1.2	22.7 ± 0.89
Fat mass (%)	14.4 ± 1.4	13.1 ± 1.6	13.5 ± 1.8	13.5 ± 2.9	12.3 ± 2.6	11.4 ± 1.6
Lean Mass (%)	74.7 ± 1.9	75.2 ± 0.8	77.1 ± 2.6	77.3 ± 3.1	76 ± 2.4	77.4 ± 1.8
Liver Weight (g)	0.83 ± 0.14	0.89 ± 0.09	0.84 ± 0.08	0.94 ± 0.07	0.99 ± 0.09	0.93 ± 0.08
Liver weight/Body weight (%)	3.59 ± 0.60	2.61 ± 0.32	3.77 ± 0.40	4.18 ± 0.26	4.28 ± 0.38	4.10 ± 0.33
Water intake (ml/kg bw/day)	181.5 ± 10.6	173.9 ± 13.6	125.5 ± 8.3	126.3 ± 10.5	122.3 ± 5.9	124.8 ± 4.1
Food Intake (kcal/kg bw/day)	142.3 ± 7.2	140.6 ± 13.1	496.0 ± 32.8	499.0 ± 41.6	483.5 ± 23.4	493.1 ± 16.4
Total Cholesterol (g/L)	0.32 ± 0.07	0.45 ± 0.09	0.40 ± 0.05	0.45 ± 0.11	0.5 ± 0.07	0.54 ± 0.06
Triglycerides (g/L)	0.28 ± 0.09	0.47 ± 0.13	0.29 ± 0.07	0.37 ± 0.08	0.4 ± 0.10	0.42 ± 0.06
NEFA (mmol/L)	0.31 ± 0.10	0.32 ± 0.29	0.15 ± 0.15	0.4 ± 0.17	0.23 ± 0.11	0.16 ± 0.04
Insuline (µg/ml)	0.69 ± 0.20	1.13 ± 0.80	1.09 ± 0.53	0.87 ± 0.29	0.52 ± 0.52	0.86 ± 0.66
Glucose (mg/dl)	139.6 ± 48.8	125.3 ± 46.5	114.7 ± 12.7	115.5 ± 17.4	135 ± 24.1	129.8 ± 19.9
Estradiol (pg/ml)	70.3 ± 13.7	56.4 ± 15.9	73.6 ± 11.2	77.1 ± 13.5	72.9 ± 11.1	76.9 ± 7.8

226 NEFA: No Esterified Fatty Acids; CTRL: control group; EPOX: epoxiconazole. Measurements were performed
 227 at 23 week-old. Values represent means ± standard deviation (SD). Data in bold and italic are significant at p <
 228 0.05 with paired Student's t-test. n = 12/group
 229

230 3.2. Multigenerational effects of epoxiconazole on hepatic metabolism:

231 ***Effect of EPOX on liver weight*** (Table 1): In males, no change in liver weight was
 232 observed whatever the generation between epoxiconazole *versus* control groups. Only in

233 female F2 mice, we observed a weak but significant increase of liver weight normalized to
234 body weight (approximately 10%, $p < 0.01$) in the EPOX group compared to the control group.

235 ***Histological liver analysis:*** We performed histological analysis of liver tissue by
236 targeting the number of Kupffer macrophages cells, fibrosis, size of hepatocytes and their
237 distribution according to their size in comparison with the control group. Our results showed
238 that an exposure to EPOX did not induce any change in Kupffer cell count and liver fibrosis
239 whatever the mouse sex and generation (supplementary data). In contrast, differences were
240 observed in the average size of hepatocytes and/or their distribution according to their size. In
241 male mice, an increase ($p > 0.01$) in the average size of hepatocytes was observed in F1 (15%)
242 and F3 (9%) mice associated with a tendency (no significant), to exhibit a proportion of larger
243 hepatocytes (Figure 1). In females, hepatocyte size was also larger in F1 (11%; $p < 0.01$) and
244 was associated with a higher proportion of larger hepatocytes (47%; $p < 0.01$). In contrast, in
245 F3 mice, although there was no difference in the average hepatocyte size, there was a
246 significant preponderance of small hepatocytes (27%; $p < 0.05$). As for males, no significant
247 difference was observed in F2 (Figure 2).

248

249

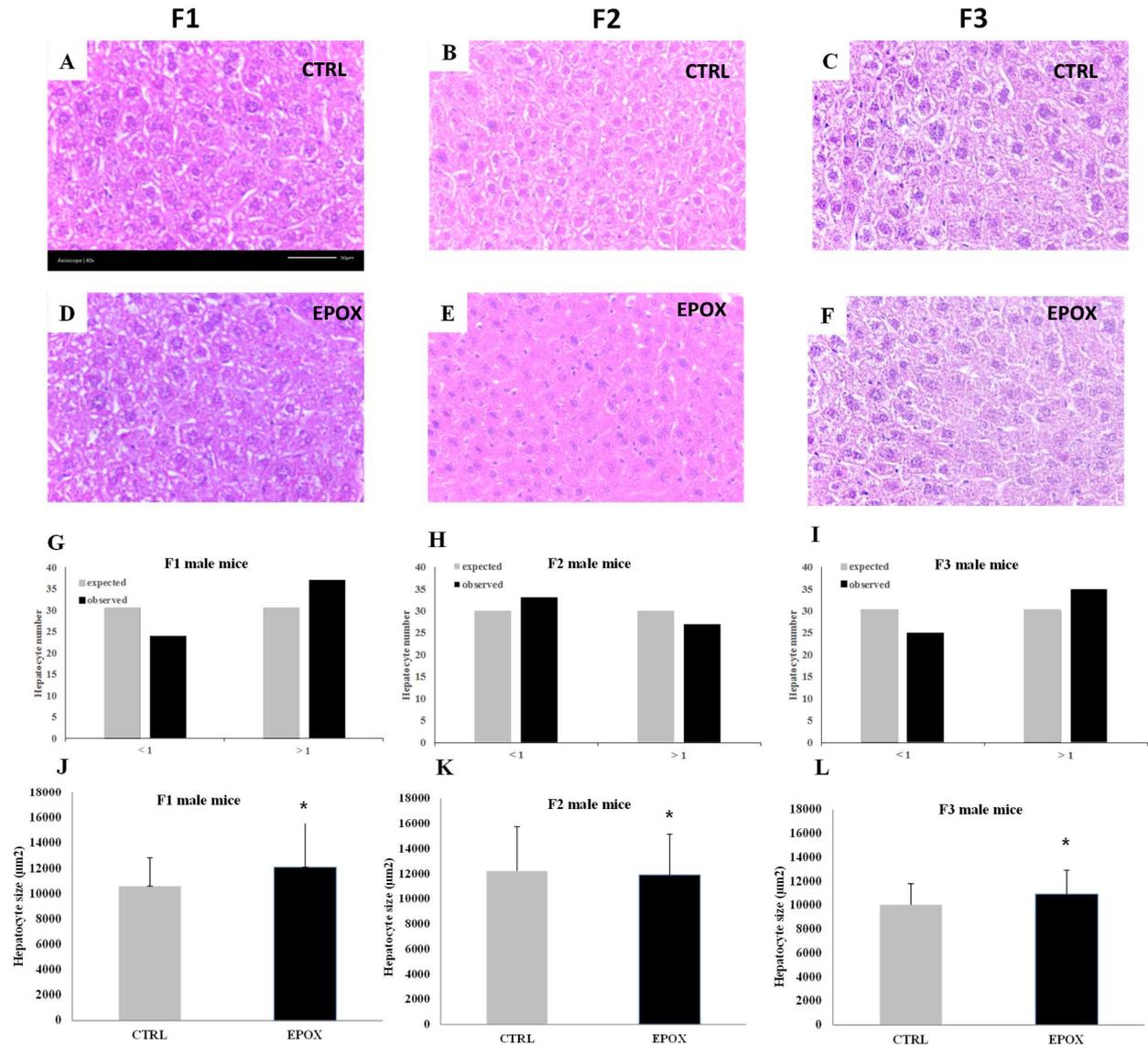
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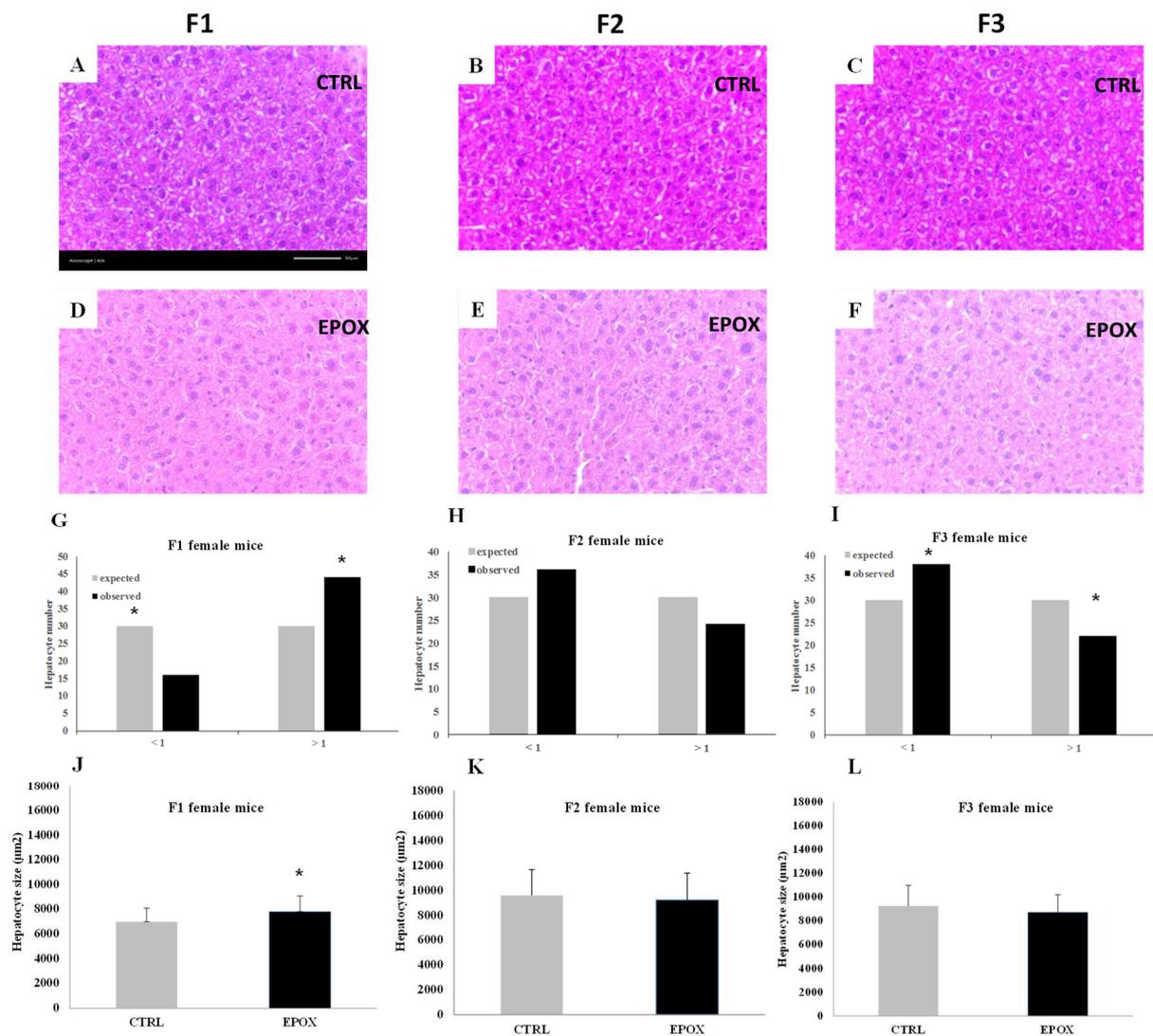
253

254



255 **Figure 1:** Transgenerational effects of epoxiconazole on the liver of male offspring
 256 from EPOX-exposed F0 females (1.75 ug/kg/d): eosin/hematoxylin stainings in control mice
 257 (CTRL; A - C) and epoxiconazole group (EPOX; D - F); distribution of hepatocytes
 258 according to their size (EPOX vs CTRL; (G - I)); size of hepatocytes (J - L). Data are
 259 expressed as the mean \pm SD. A Student's t-test or a Chi-square test has been achieved; * p
 260 <0.05; compared to controls for each generation. n = 12/group. Magnification x40.

261
 262
 263



264 Figure 2: Transgenerational effects of epoxiconazole on the liver of female offspring from
 265 EPOX-exposed F0 females (1.75 ug/kg/d): eosin/hematoxylin stainings in control mice
 266 (CTRL; A - C) and epoxiconazole group (EPOX; D - F); distribution of hepatocytes
 267 according to their size (EPOX vs CTRL; (G - I)); size of hepatocytes (J - L),. Data are
 268 expressed as the mean \pm SD. A Student's t-test or a Chi-square test has been achieved; * p
 269 <0.05; compared to controls for each generation. n = 12/group. Magnification x40.

270

271

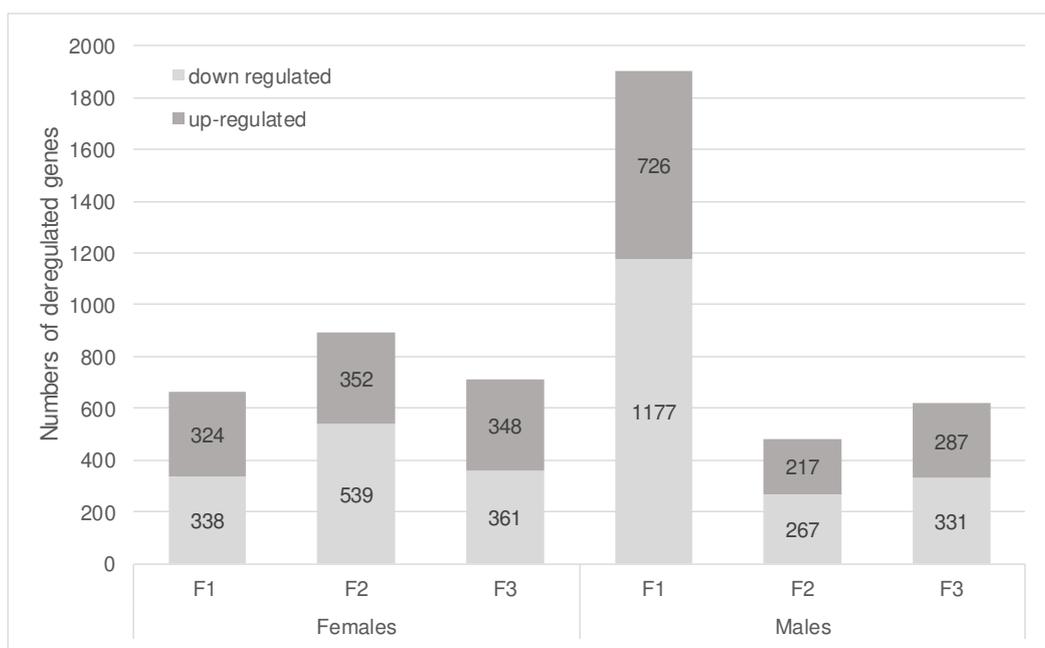
272

273 3.3. Transcriptomic analysis in liver

274 To clarify the molecular mechanisms underlying and associated with the
 275 transgenerational hepatic effects of EPOX, we performed a transcriptomic analysis. The
 276 observed differential mRNA expressions were put in perspective of the associated biological
 277 systems by an analysis with KEGG databases. To refine the analysis of the genes whose
 278 expression is altered, comparisons between generations and between sexes of individuals
 279 were performed.

280 3.3.1 Transgenerational effect of EPOX by gender and generation

281 Using a threshold of 1.5-fold and a p value <0.05, a significant number of genes among the
 282 22,206 genes showed an alteration of their mRNA expression level with a preponderance of
 283 down-regulated genes (supplementary data table S2 A, B and C for male mice and S2D, E and
 284 F for female mice). A difference according to the sex was observed with a profile evolving
 285 over the generations (Figure 3). F1 male mice were the most impacted with 1,903 deregulated
 286 genes of which 1,177 (61.8%) were down-regulated. In females, F2 mice exhibited the most
 287 altered transcriptomic profile with 891 deregulated genes of which 539 (60.5%) were down-



288 regulated.

289 Figure 3: Number of genes with mRNA expression significantly altered (at least 1.5-fold) in
290 female and male mice of F1, F2 and F3 generations came from epoxiconazole-treated dams
291 F0 in comparison with respective control group.

292 3.3.2 Functional gene set enrichment analysis (GSEA)

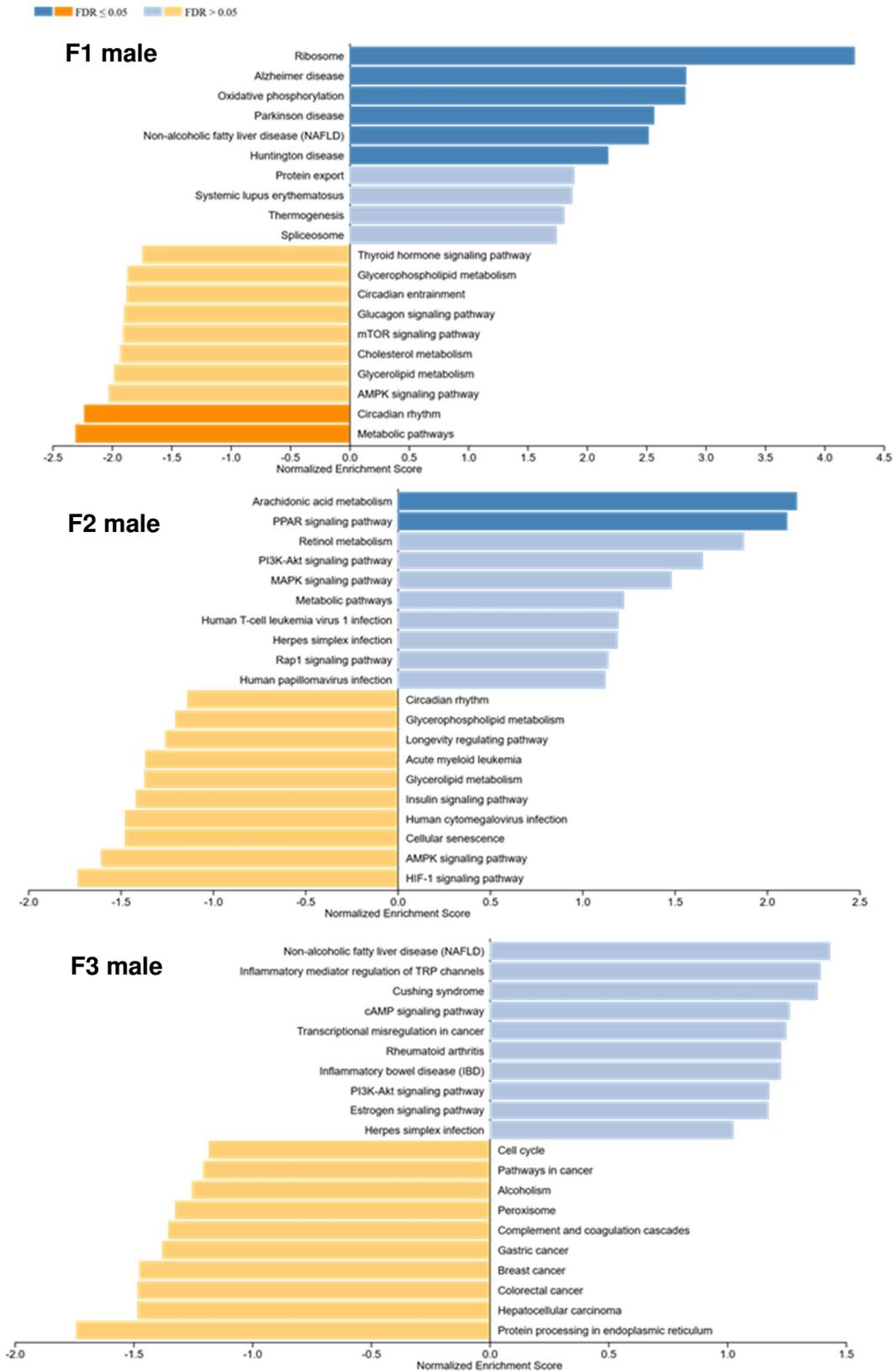
293 GSEA was performed with WebGestalt 2019 using the KEGG pathway databases.
294 Biological systems associated with EPOX exposure also differed by sex and generation. In F1
295 male mice, genes associated with increased ribosome activity, oxidative phosphorylation, and
296 dysregulation leading to NAFLD were significantly represented (Figure 4 and Supplementary
297 data table S1 for the list of concerned gene). Conversely, the systematic keywords, “circadian
298 rhythm” and “metabolic pathway” emerged significantly down-regulated. Concerning F2
299 males, two systems still appear significantly active ($FDR < 0.05$), arachidonic acid metabolism
300 and the PPARs signaling pathway while there is nothing statistically significant in F3.

301 In females, the olfactory transduction pathway appears to be activated in both F1 and
302 F2, but the genes involved are not the same and are fewer in F2. In F3, only the PPARs
303 signaling pathway is significantly ($FDR < 0.05$) down-regulated (Figure 5 and Supplementary
304 data table S1 for the list of concerned gene).

305 3.3.3 Comparison of transcriptomic profile over the generations and the gender

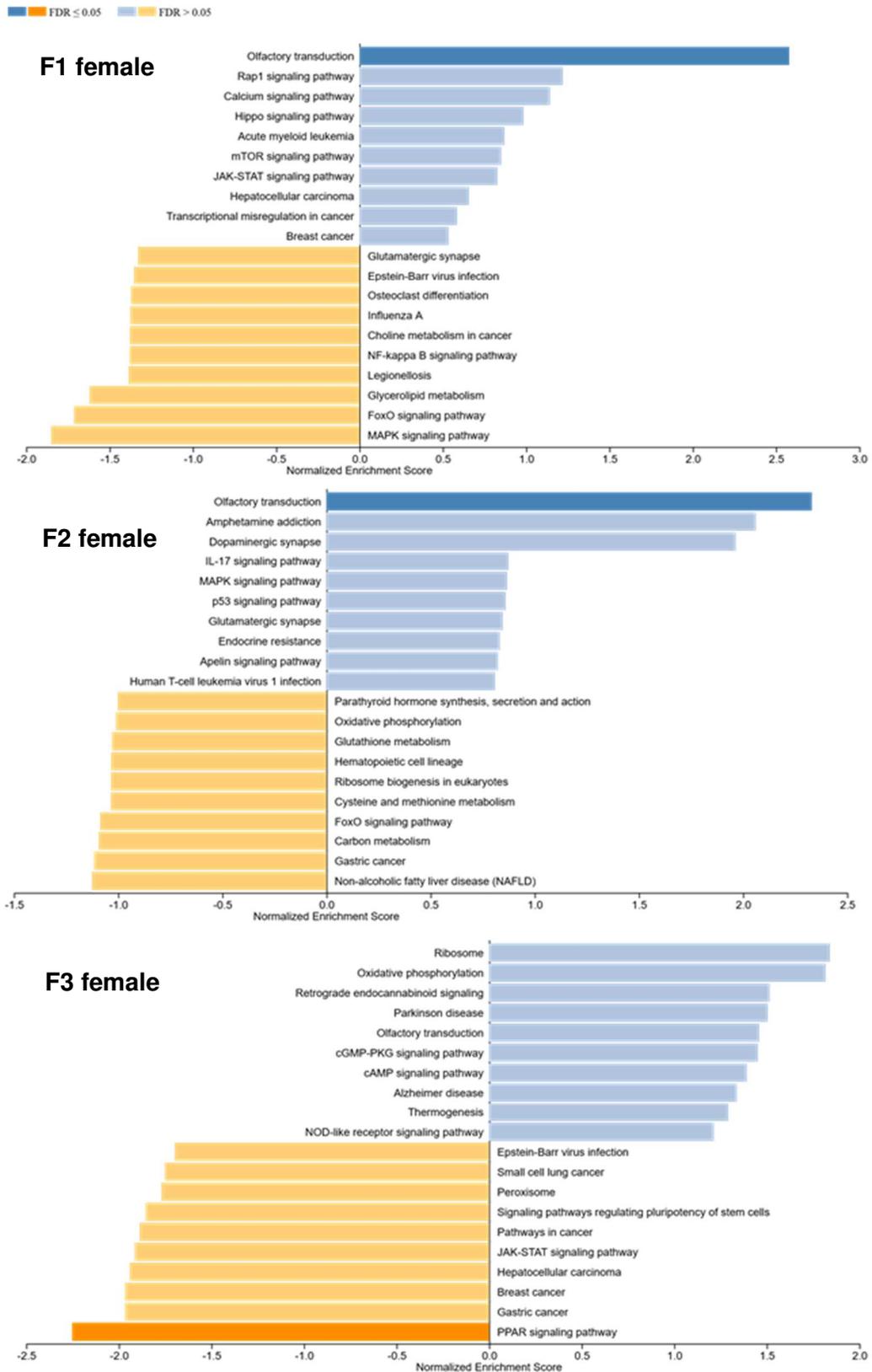
306 A comparative analysis of deregulated genes between generations was performed
307 (Figure 6). The common differentially expressed genes among generations were then
308 analyzed using String (<http://string-db.org/>). The analysis showed that in both males and
309 females, the circadian rhythm is the system that is mainly altered. More specifically, in F1 and
310 F2 males, there are also genes involved in energy metabolism that are deregulated, notably in
311 the metabolism of glycerolipids, glycerophospholipids, insulin sensitivity and those
312 associated with the development of type 2 diabetes. Differential mRNA expression of these
313 genes is largely identical between F1 and F2 generations in males and females. In contrast,

314 genes common to the F2 and F3 generations showed a variation in mRNA expression in
315 opposite directions (*e.g.*, the *ciart* gene is upregulated in F2 and downregulated in F3;
316 supplementary data Table S2G for male mice and S2H for female mice).



317 Figure 4: Functional Gene Set Enrichment Analysis identified by the databases of pathways
 318 KEGG for down- and up-regulated genes in the livers of male mice from epoxiconazole-

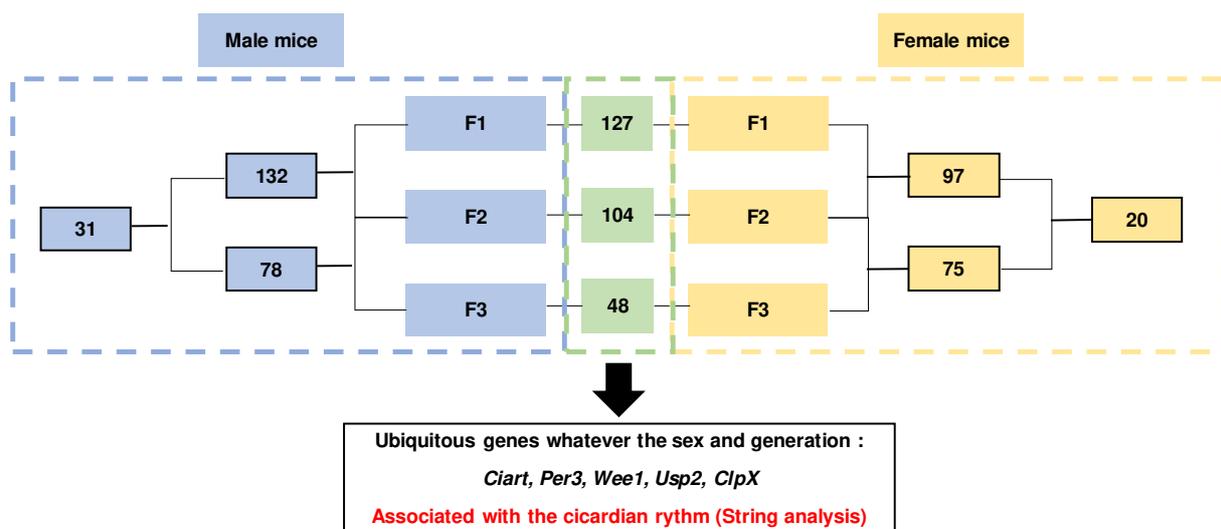
319 treated F0 dams in comparison with control mice for each generation. n = 3 pools of 3
320 animals.



321 Figure 5: Functional Gene Set Enrichment Analysis identified by the databases of pathways
 322 KEGG for down- and up-regulated genes in the livers of female mice from epoxiconazole-

323 treated F0 dams in comparison with control mice for each generation. n = 3 pools of 3
 324 animals.

325 A comparison between generation of the altered genes (Figure 6 and Supplementary
 326 Data S2I) indicates the same findings for females and males, namely an impact on circadian
 327 rhythm and metabolic pathways. Interestingly, the mRNA expression profile of these common
 328 genes is almost similar depending on the sex or generation considered.



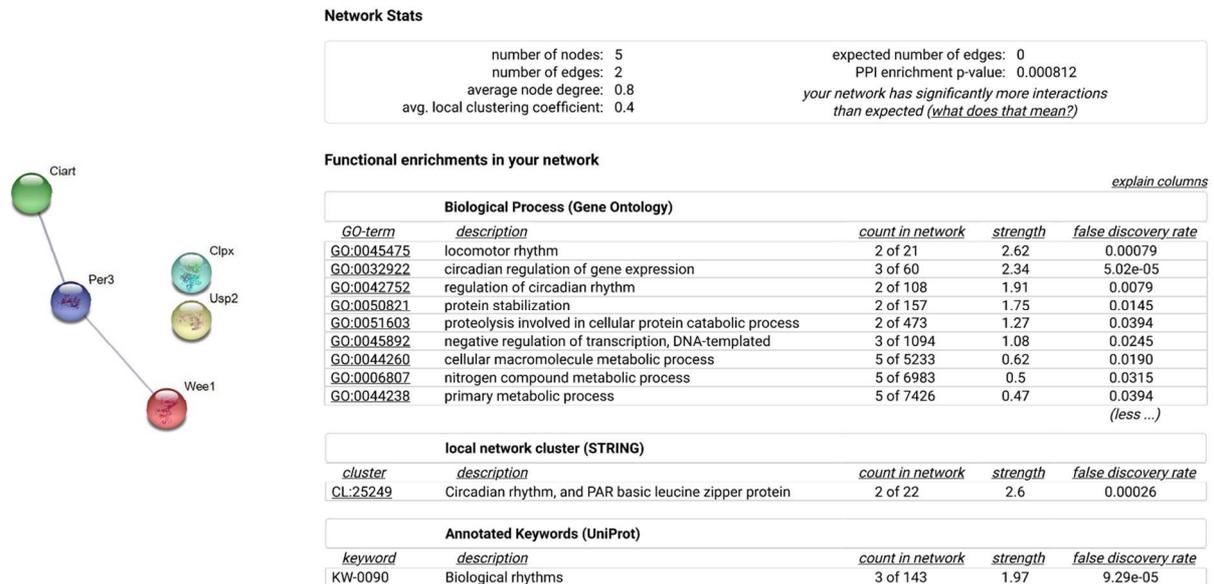
329 Figure 6: Number of genes with altered mRNA expression and that are common across
 330 generations and/or sex of the individual. In the yellow boxes, the values for females and in the
 331 blue boxes, those for male mice. In the green boxes is indicated the number of genes common
 332 between the two sexes and for each generation, respectively. Only five genes are common to
 333 all generations and to both males and females.

334

335 Compiling all these data, the alteration of the expression of five genes appears to be
 336 conserved over the generations independently of the individual sex: *Circadian Associated*
 337 *Repressor of Transcription (Ciart)*, *Period Circadian Regulator 3 (Per3)*, *WEE 1 homolog 1*
 338 *(Wee1)*, *Ubiquitin carboxyl-terminal hydrolase 2 (Usp2)* and *caseinolytic mitochondrial*
 339 *matrix peptidase chaperone subunit (Clpx)*. Apart from this last gene, the other four are
 340 associated with the control of the circadian rhythm (Figure 7).

341

342



343 Figure 7: String (string-db.org) analysis with the five genes commonly altered
 344 whatever the sex and the generation of mice from EPOX-treated F0 dams in comparison with
 345 control group.

346 4. Discussion

347 4.1 Originality and limitations of the study

348 **Originality and contribution to the field:**

349 Studies investigating the hepatotoxicity of EPOX are obviously necessary and of great
 350 interest, but they do not take into account two essential elements: the concept of low dose
 351 exposure (closer to reality) and the developmental origins of health and disease (DOHAD).
 352 This concept of DOHAD has greatly modified our understanding of what makes an individual
 353 develop or not, in the course of his life, the main chronic diseases that affect rich or emerging
 354 countries. It implies a shift in the paradigm on which prevention policies can be based on a
 355 global scale, in the major field of epidemiology and mechanisms of non-communicable
 356 diseases according to the World Health Organization classification (Desai et al., 2015). Thus,

357 based on a multigenerational study, our objective was to characterize the hepatotoxic effects
358 of perinatal low-dose exposure of EPOX, particularly at the molecular level, and the
359 consequences in the descendants.

360 *Limitations of the study*

361 The analysis of this type of study is complex because it incorporates several factors of
362 physiological importance: the sex of the animal, the generation, the exposure, the dose and the
363 substance studied. In order to characterize the effect of EPOX, the protocol was based on a
364 previous laboratory study on another endocrine disruptor, bisphenol S (Brulport et al., 2021).
365 This exposure protocol allows for the critical exposure windows of embryonic and fetal
366 development and the lactation period. It also enables the study of transgenerational effects
367 that may be observed in the third generation, which is the first generation not exposed to the
368 substance, even indirectly *via* germ cells (as is the case for F2 generation).

369 The comparison with previous studies on the hepatotoxicity of EPOX is not very
370 relevant because of the differences in the experimental strategy implemented in our study.
371 Indeed, to date and to our knowledge, this is the first time that the transgenerational hepatic
372 effects of EPOX, or evenazole compounds, have been studied notably after gestational
373 exposure.

374 The use of ethanol as vehicle for EPOX exposure may raise questions about these
375 possible effects. Few studies have investigated the effect of ethanol exposure at low-dose
376 during the gestation and lactation in laboratory mice, notably through drinking water. Allan *et*
377 *al.* (2003) showed that maternal food consumption and care, and litter size and number were
378 all found to be similar for the alcohol-exposed and control animals. Only an increase of the
379 time spent inspecting a novel object introduced into the open field was observed. In their
380 study, the average daily consumption of ethanol by mouse dams on the 10% ethanol solutions
381 was 14.0 g ethanol/kg bw/d (about 7.2 mg ethanol/kg bw/d in our study). Without obscuring

382 the consequences of even small amounts of ethanol exposure, we also observed no significant
383 differences in litter size or neonate weight (CTRL 1.38 ± 0.16 g vs EPOX 1.40 ± 0.14 g for
384 F1; CTRL 1.31 ± 0.08 g vs EPOX 1.34 ± 0.09 g for F2; CTRL 1.36 ± 0.13 g vs EPOX $1.35 \pm$
385 0.11 g for F3; values represent means \pm SD for neonates body weight at PND0). Similarly, the
386 use of isoflurane to anaesthetize mice prior to euthanasia could potentially affect the
387 expression of genes encoding cytochrome P450. Indeed, both ethanol and isoflurane have
388 been described to induce CYP2A4 activity and mRNA expression (García-Suástegui et al.,
389 2017). However, Plate *et al.* (2005) also reported that rats can be anaesthetized with isoflurane
390 for a short period without a change in the activity of P450 enzymes (Plate et al., 2005). In
391 order to avoid possible consequences, the time between the application of isoflurane and liver
392 sampling was kept as short as possible.

393 4.2 Physiological and histological consequences

394 Hamdi et al. (2019) described that EPOX induced significant histopathological
395 changes (necrosis, dilatation and congestion of the central vein and portal triad, dilation of
396 sinusoidal capillaries, lipid vacuolization, hemorrhage, and inflammatory leukocyte
397 infiltration) in male Wistar rats exposed to 8, 24, 40 and 56 mg/kg bw/d for 28 days, with
398 induction of oxidative stress associated with changes in liver biomarkers ALT, AST, ALP,
399 LDH (Hamdi et al., 2019). In our study, changes in liver histology were also observed but we
400 failed to identify transcriptomic changes indicating a link to oxidative stress. The oxidative
401 activity of EPOX occurs mainly at high concentrations both *in vivo* and *in vitro* (10^{-5} M in
402 human colon carcinoma cells (HCT116) for example (Hamdi et al., 2018)) which may explain
403 its absence in our experimental conditions.

404 In male CD1 mice, Hester et al. reported that exposure for 30 days to EPOX doses
405 ranging from 1 to 500 μ g/kg bw/d (carcinogenic doses) induced hepatic effects such as CYP
406 induction, hepatic hypertrophy, hepatomegaly, increased cell proliferation, and decreased

407 serum cholesterol levels. Likewise, Heise *et al.* (2015, 2018) reported the effects of three
408 widely used hepatotoxic (tri)azole fungicides (cyproconazole, epoxiconazole, and prochloraz)
409 in a 28-day study in male rats. The three fungicides were administered individually or in
410 mixture at doses ranging from 0.9 to 1000 $\mu\text{g}/\text{kg}$ bw/d. Their results showed, at the highest
411 dose, an increase in liver weight associated with histopathological changes, such as increased
412 hepatocyte size and vacuolation (Heise *et al.*, 2018, 2015). These results are quite similar to
413 our results, except for the dose necessary to induce an observable effect. This difference is
414 probably related to the gestational exposure in our study. These data underline the importance
415 of the exposure window to harmful substances, especially during the critical phases of
416 development.

417 In F3 males, although no biological system stands out significantly, the
418 transgenerational consequence of EPOX exposure appears to be much more global as the F3
419 male mice have a lower body weight than the control group, but no change in their body
420 composition (lean and fat mass). These results suggest a smaller body size of these animals,
421 which also associated with a decrease in food and water intake. The reasons for these
422 physiological consequences are unclear and need to be further investigation.

423 4.3 Key pathway associated with effect of EPOX: sex and generation dependent

424 To evaluate the consequences of the EPOX exposure in the liver, through mRNA
425 expression, we set to a hypothesis-free approach. Ontological analysis allows to highlight the
426 biological systems associated with the observed transcriptomic profile. In general, these
427 perturbations are signs of a strongly impacted metabolic activity.

428 *Hepatic metabolism*

429 Male F1 mice are the most affected by the hepatotoxic effect of EPOX with a
430 stimulation of protein synthesis, oxidative phosphorylation and metabolic dysregulation
431 associated with the development of NAFLD. This hepatic metabolic activity is confirmed by

432 the increase in hepatocyte size observed in these animals. Indeed, hepatocyte size is
433 influenced by a number of factors such as regenerative activity and metabolic status. For
434 example, the influx of solutes such as amino acids or glucose causes transmembrane
435 movement of water into the cells, increasing their volume by 5-10% (Saxena, 2018).
436 Increased glycogen or lipid stores may also impact on hepatocyte size. Although we did not
437 measure hepatic triglycerides, an analysis of the rate of lipid infiltration (rough indicator) on
438 liver HE/eosin sections by J-image showed no significant difference (data not shown).

439 *Arachidonic acid pathway*

440 The arachidonic acid pathway appears to occur mainly in F2 male. It may indicate an
441 inflammatory state that is not confirmed by histological analysis, particularly at the level of
442 fibrosis.

443 *Olfactory transduction*

444 Unexpectedly, in F1 and F2 female mice, GSEA showed an involvement of the gene
445 encoded products in olfactory transduction with 31 olfactory receptor genes up-regulates. *Olf*
446 genes are the largest gene superfamily in vertebrates with 1,296 mouse *Olf* genes (including
447 20% pseudogenes) reported and classified into 228 families (Zhang and Firestein, 2002).
448 Initially, these G protein-coupled receptors are described as recognizing and sensing volatile
449 chemicals serving as exogenous ligands to mediate odor perception in the brain (Fleischer et
450 al., 2009). These receptors are also ectopically expressed in other tissues such as testis, lung
451 and liver, where they are also functional and activated by endogenous ligands (Kang and Koo,
452 2012; Maßberg and Hatt, 2018). Even if the role of *Olf* receptors in the liver is not known
453 yet, some recent studies have highlighted their involvement in hepatic energy metabolism. For
454 example, olfactory receptor 544 (*Olf*544) is highly expressed in mouse liver and adipose
455 tissue, where it has been implicated in the metabolic interaction between liver and adipose
456 tissue. *Olf*544 leads to the mobilization of fat stored in the adipose tissue and the shift of

457 energy preference towards liver and brown adipose tissue (Wu et al., 2017). Likewise, in
458 mouse livers and cultured mouse hepatocytes, Olfr43 activation was associated with a
459 reduction in hepatic triglyceride accumulation in the mouse liver (Wu et al., 2019). Olfr
460 receptors have also been described to be involved in glucose metabolism. For example, by
461 using Olfr734 deficient mice, Li *et al.* (2019) reported that this receptor functioned as an
462 Asprosin receptor to regulate hepatic glucose production. Asprosin is a fasting-induced
463 hormone that stimulates glucose production in the liver and appetite at the hypothalamic level
464 (Li et al., 2019). Although the biological significance of the GSEA results must be interpreted
465 with caution, the regulation of 31 chemosensory receptor-encoding genes in mouse liver
466 strongly suggests that EPOX has the potential to modulate the liver metabolism. Future
467 studies are needed to clarify the biological consequences of the regulation of a large number
468 of chemosensory receptor genes in the liver.

469 ***PPAR α pathway***

470 In F3 females, olfactory transduction still appears in the GSEA, but not significantly
471 (FDR>0.05). In contrast, some genes associated with the PPAR signaling pathway were
472 downregulated. PPAR α regulates lipid metabolism in the liver as a major control of fatty acid
473 oxidation and energy generation in a fasted state (Wang et al., 2020). Decreased stimulation of
474 the PPAR α signaling pathway may thus be associated with hepatocytes exhibiting less
475 metabolic activity, which correlates with the higher proportion of smaller hepatocytes
476 observed in the present study. However, it is also noteworthy that some genes for ribosomal
477 and oxidative phosphorylation processes tend to be upregulated, but in a non-significant
478 manner (FDR>0.05). A stimulation of protein synthesis and oxidative phosphorylation can
479 therefore not be excluded. This can be explained by the cellular specificity of PPAR α
480 activities and the diversity of hepatic cells. The liver is composed of 80% hepatocytes and
481 20% non-parenchymal cells (Kupffer cells, hepatic sinusoidal endothelial cells and hepatic

482 stellate cells) involved in intercellular trafficking and immune regulation (Werner et al.,
483 2015). Thus, each cell type may respond differently to EPOX exposure. Indeed, it is unclear
484 which hepatic cell type is principally involved in PPAR α function in lipid homeostasis and
485 phenotypes such as hepatomegaly and hepatocyte proliferation. For example, cell
486 proliferation has been described as mediated exclusively by PPAR α activation in hepatocytes,
487 whereas Kupffer cell PPAR α has an important role in the mediation of anti-inflammatory
488 effects of PPAR α agonists (Brocker et al., 2017).

489 Activation of the PPAR pathway may also be an indicator of inflammation. In
490 accordance with these data, in these male F2 mice, we observed an enhancement of mRNA
491 expression for *Cyp4a31* (1.94-fold), *Cyp4a32* (2.21-fold) and *Cyp4a14* (11,91-fold). The
492 significant increase of *CYP4a* gene expression is associated with PPAR-dependent liver
493 inflammation. (Mitchell et al., 2001). Members of the CYP4A subfamily catalyze the
494 hydroxylation of fatty acids such as arachidonic acid (Nguyen et al., 1999) and Sharma *et al.*
495 demonstrated that their expression in rodent livers is induced by PPAR nuclear receptors
496 (Sharma et al., 1989). Interestingly, a characteristic of hepatic CYP4A induction by PPAR in
497 this species is a marked sex difference, females being refractory (Sundseth and Waxman,
498 1992).

499

500 4.4 Transcriptomic transgenerational effect of EPOX

501 Mutual genes in males and females showed similar expression variations between F1
502 and F2 and complete opposition between F2 and F3. It seems that molecular mechanisms are
503 set up during the generations to counterbalance the effects of EPOX exposure in F0 pregnant
504 females. In this study, these variations in gene expression observed in F3 are still associated
505 with physiological disorders either at the individual level (e.g. body weight of F3 males) or at
506 the histological and transcriptomic levels (F3 females). Taken together, these data indicate

507 that EPOX induces a transgenerational effect in a sex-dependent manner. Many mechanisms
508 by which exposure to harmful substances could lead to liver disease have been identified
509 (Foulds et al., 2017). For example, correlations between endocrine disruptor exposures during
510 the development and changes in gene expression are associated with DNA methylation,
511 histone code pattern variations, or interference with microRNAs (Barouki et al., 2018).

512 4.4 The circadian rhythm: a key factor of the EPOX effect across generations?

513 Comparison of genes between males and females in each generation indicates that
514 some genes are similarly altered in all generations. Surprisingly, these genes are involved in
515 controlling the circadian rhythm of gene expression. As described in the results section, the
516 five genes *Ciart*, *Wee1*, *Per3*, *Clpx*, and *Usp2* are the only ones that have their expression
517 altered regardless of sex or generation.

518 The circadian clock system includes the central clock in the suprachiasmatic nucleus
519 of the hypothalamus and peripheral clocks located in peripheral tissues. At the hepatic level,
520 they are involved in the hepatic homeostasis by playing an important role in the regulation of
521 energy metabolism and the expression of enzymes regulating the absorption and metabolism
522 of xenobiotics. Dysregulation of the liver clock is involved in the development of liver
523 diseases such as fatty liver disease, cirrhosis, hepatitis and liver cancer, and these disorders
524 also disrupt the function of the liver clock (Tahara and Shibata, 2016).

525 Many genes related to liver metabolism (lipogenesis, gluconeogenesis, mitochondrial
526 activity, bile acid synthesis and oxidative metabolism) show circadian variation in their
527 expression (Bass and Takahashi, 2010). In mice, alteration of the core clock genes results in
528 metabolic disorders. Interestingly, mice with a *Clock* gene mutation (*Clock Δ 19/ Δ 19*) had a
529 decrease of insulin plasma level and an increase of cholesterol and triglyceride plasma levels
530 in comparison with wild-type mice (Marcheva et al., 2010). In concordance with our results
531 showing the involvement of PPAR pathway, it was reported that PPAR nuclear receptors are

532 important regulators of the circadian clock system and subsequently of energy metabolism
533 (Schmutz et al., 2010; Tahara and Shibata, 2016). Likewise, many CYP phase I superfamily
534 mRNAs, including Cyp4a14, are expressed rhythmically in mice, with enhanced levels during
535 the dark phase (Zhang et al., 2009). Thus, the sex and generation-dependent disturbances
536 appear to be inseparable from a deregulation of the circadian rhythm, which is sex and
537 generation-independent. At this stage, it is obviously impossible to determine the causal link
538 of these mechanisms.

539 *EPOX induce a gender-dependent transgenerational effect*

540 From a physiological and transcriptomic point of view, EPOX induce sex-dependent
541 effects in mice. This discordance suggests sex hormone-dependent effects potentially related
542 to the estrogenic and anti-androgenic properties of EPOX, both in the activity and synthesis of
543 these hormones (DAR, 2006). In agreement with these data, in our study, EPOX induced a
544 decrease in plasma estradiol levels in F1 females, which has been previously reported in GD
545 21 Wistar dams, but exposed to a high dose of EPOX (50 mg/kg/bw) (Taxvig et al., 2008).
546 Decreased estrogen levels are classically associated with increased body fat (Mauvais-Jarvis
547 et al., 2013), which is the opposite in the present study. These results therefore suggest that
548 other mechanisms may be involved, particularly at the level of adipose tissue. Indeed, as
549 indicated by the increase in plasma triglyceride levels, the lipolytic activity and/or the uptake
550 of triglycerides by adipose tissue could be impacted (Beigneux et al., 2007; Hajer et al.,
551 2008).

552 These sex-dependent effects could also result, in part, from a different toxicokinetic of
553 EPOX between males and females. The metabolism of EPOX is rapid and extensive with the
554 production of at least 47 metabolites in rat. However, its elimination is slow and no major
555 differences in the metabolism of epoxiconazole in rats were observed with regard to sex and
556 dose level. However, based on urinary and biliary elimination, oral bioavailability was

557 approximately 80% in males and 50% in females (DAR, 2006). This concept would only
558 concern mice of the F1 generation, which is the only one potentially exposed during the
559 lactation.

560

561 **5. Conclusion**

562 Hepatotoxicity of epoxiconazole has been shown following high dose exposure in adult
563 rodent. In this study, we demonstrate that this fungicide is able to induce hepatic perturbations
564 in mice following a low dose perinatal exposure. These effects also show a transgenerational
565 pattern with strong disparities according to the sex and generation of the animals. This study
566 also highlights the potential involvement of a disruption of the hepatic circadian rhythm. The
567 data obtained indicate a likely alteration of the inflammatory status of the liver, but with no
568 observable histological consequences at the age of the animals in this study. As a consequence
569 of these effects at the transcriptional level, the question of the long-term development of more
570 severe liver pathologies (NASH, NAFLD, cirrhosis, cancer) remains to be studied.

571 **6. Declaration of competing interest.**

572 The authors declare that they have no known competing financial interests or personal
573 relationships that could have appeared to influence the work reported in this paper.

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580

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