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1 **Perinatal exposure to chlorpyrifos and/or a high-fat diet is associated with liver**
2 **damage in male rat offspring**

3

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17

18 Declarations of interest: none

19 **List of abbreviations:**

20

21 *Acaca*: Acetyl-CoA carboxylase 1

22 *Actb*: Beta actin

23 ALAT: Alanine aminotransferase

24 ALP: Alkaline phosphatase

25 ASAT: Aspartate aminotransferase

26 BW: Body weight

27 *Cd36*: Fatty Acid Translocase

28 cDNA: Complementary DNA

29 *Col1a1*: Collagen alpha-1

30 CPF: Chlorpyrifos

31 *Dgat1*: Diglyceride O-acyltransferase 1

32 *Dgat2*: Diglyceride O-acyltransferase 2

33 DOHaD: Developmental Origins of Health and Disease

34 *Fasn*: Fatty acid synthase

35 *Gapdh*: Glyceraldehyde 3-phosphate dehydrogenase

36 *G6pc*: Glucose-6-phosphatase

37 HDL; High-density lipoprotein

38 HF: High-fat diet

39 *Hmgcr*: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase

40 *Hprt1*: hypoxanthine guanine phosphoribosyl transferase 1

41 IGF1: Insulin-like growth factor-1

42 *IL1b*: Interleukin 1 beta

43 *Lpl*: Lipoprotein lipase

44 LDH: Lactate dehydrogenase

45 *Mlxipl/Chrebp*: Carbohydrate response element binding protein

46 NOAEL: No observable adverse effect level

47 OP: Organophosphate

48 PAS: Periodic acid Schiff

49 *Pck1/Pepck*: Phosphoenolpyruvate carboxykinase

50 PND: Postnatal day

51 *Ppara*: Peroxisome proliferator-activated receptor alpha

52 *Pparg*: Peroxisome proliferator-activated receptor gamma

- 53 *Prkaa1/Ampka*: AMP-activated protein kinase catalytic subunit alpha
- 54 RT-PCR: Reverse transcription polymerase chain reaction
- 55 SC: Standard chow diet
- 56 SEM: Standard error of the mean
- 57 *Slc2a2/Glut2*: Solute carrier family 2, facilitated glucose transporter member 2
- 58 *Srebf1/Srebp1c*: Sterol regulatory element binding factor 1 variant 2
- 59 TC: Total cholesterol
- 60 TG: Triglyceride
- 61 T2DM: Type 2 diabetes
- 62 *Ubc*: Ubiquitin C
- 63 VLDL: Very-low-density lipoprotein.

64 **Abstract:**

65

66 Metabolic impairments in childhood are known to promote the development of type 2
67 diabetes and/or obesity in adulthood. These impairments may result from perinatal
68 exposure to harmful environmental factors, such as pesticide residues or the
69 consumption of a “western” diet. In the present study, we sought to determine
70 whether an obesogenic profile, metabolic disorders and liver damage in offspring
71 (observed during young adulthood) were related to maternal exposure to the
72 pesticide chlorpyrifos (CPF) and/or a high-fat diet (HFD) starting 4 months before
73 conception and ending at weaning. After the end of exposure, 51 male rat pups were
74 left to develop under normal conditions and were studied in young adulthood. Despite
75 the absence of direct exposure to harmful factors (other than through the dam’s milk),
76 maternal exposure to CPF or an HFD was associated with changes in the offspring’s
77 metabolic activity in the liver in the offspring. This indirect exposure to CPF was
78 associated with a relative reduction in the expression of genes coding for enzymes
79 involved in lipid or glucose metabolism but did induce histopathological changes in
80 the offspring at adulthood. Maternal exposure to an HFD alone or to CPF alone gave
81 similar results in offspring, changes in the same direction. Exposure of the mother to
82 HFD did not exacerbate CPF effects. Co-exposure to both CPF and HFD did not
83 increase the observed effects compared to each factor taken separately.

84

85 **Keywords:** Chlorpyrifos; High-fat diet; Maternal exposure; Metabolic diseases; Liver;

86 Rats

87

88

1. Introduction

89

90 The prevalence of obesity or overweight in children is a serious, growing public
91 health concern and is associated with greater morbidity and mortality in early
92 adulthood. There is a genetic predisposition to obesity/overweight: parental obesity
93 doubles the risk of adult obesity in children under the age of 10 (Whitaker et al.,
94 2009) and is therefore a major risk factor in this context. The biological mechanisms
95 that underlie these effects have not yet been fully elucidated, although a growing
96 body of evidence suggests that environmental factors during pregnancy may
97 modulate foetal growth and development and thus predispose the offspring to
98 metabolic diseases in later life (for a review, see (Sarron et al., 2020)). These
99 environmental factors include pesticides, i.e. substances used to control organisms
100 that are harmful for plants and animals in agriculture. Even though the use of
101 pesticides increases yields in agriculture, it is increasingly clear that these compounds
102 have detrimental health effects - particularly during highly sensitive periods of life.
103 The perinatal period comprises several highly plastic developmental phases, during
104 which sensitivity to environmental factors is at its highest. The maternal environment
105 (including exposure to chemical agents) might therefore influence foetal growth. Most
106 of these factors are likely to influence growth directly through changes in nutrient
107 transfer or damage to the thyroid gland, or indirectly through foetal immunotoxicity
108 and neurotoxicity (Kamai et al., 2019). Indeed, prenatal pesticide exposure is likely to
109 contribute to the occurrence of obesity and type 2 diabetes later in life, exemplifying
110 the “Developmental Origins of Health and Disease” concept. Organophosphates
111 (OPs) are still the most widely used category of pesticides worldwide, although their
112 known consequences on both animal and human health means that use in France
113 and other countries is currently decreasing. The OPs’ main mechanism of action is
114 the irreversible inhibition of acetylcholinesterase activity in both insects and
115 mammals. However, other mechanisms are probably involved in the long-term
116 effects of foetal or neonatal OP exposure (Slotkin, 2004). Most human exposure is
117 due to the oral ingestion of pesticide residues present on fruits and vegetables. Since
118 OPs and their metabolites cross the placenta easily, a foetus can be exposed
119 throughout pregnancy (Fish, 1966). The OP chlorpyrifos-ethyl (CPF) has been widely
120 used to protect cereal crops against pests. However, CPF’s use has been questioned
121 because a large body of evidence shows that this compound can disrupt embryonic

122 development (Slotkin et al., 2005b) and affect the foetal nervous system (Hunter et
123 al., 1999; Akhtar et al., 2006) - phenomena that have repercussions in postnatal life
124 (Slotkin, 1999). Early-life CPF exposure alters glucose and lipid metabolism
125 (culminating in prediabetes) (Slotkin, 2011) and induces oxidative stress and lipid
126 peroxidation. The persistence of these alterations into adulthood suggests that
127 adverse effects can be transgenerational (Ndonwi et al., 2019). This type of exposure
128 is a known risk factor for obesity (Lassiter and Brimijoin, 2008) and is responsible for
129 maternal and developmental toxicity (Maurissen et al., 2000). We have reported
130 previously that repeated, low-level, oral maternal exposure to CPF was altering foetal
131 development resulting in long-term neurological effects on the offspring with later
132 consequences in adulthood (Laporte et al., 2018). We also showed that CPF
133 exposure during gestation and development has both short- and long-term impacts
134 on the digestive tract (Joly Condetta et al., 2015). Furthermore, we suggested that
135 various types of pre- and post-natal exposure could impair adult metabolism and
136 biochemical status and thus predispose the individual to metabolic syndrome. This
137 hypothesis was developed further in a recent review; the researchers hypothesized
138 that OP exposure is hepatotoxic, induces inflammation and oxidative stress, and
139 leads to hyperglycaemia and other impairments in glucose metabolism (including
140 increased glycogenolysis and gluconeogenesis) thus directly associating OPs with
141 obesity and diabetes (Czajka et al., 2019). In particular, CPF exposure was shown to
142 impair lipid metabolism and induce histopathological changes in the liver (Elsharkawy
143 et al., 2013).

144 Furthermore, the mother's diet and body composition before pregnancy have a great
145 influence on her child's future health (Barker, 2003). A study of 97 overweight or
146 obese pregnant women found the latter's children developed fatty liver in young
147 adulthood (Cantoral et al., 2020). This effect has also been observed in the
148 laboratory, since long-term maternal consumption of a high-fat diet (HFD), mimicking
149 dietary habits in Western societies (Srinivasan et al., 2006) starting before
150 pregnancy and lasting until weaning was associated with metabolic syndrome
151 phenotypes in the rat offspring (Tellechea et al., 2017). Taken as a whole, these data
152 suggest that maternal obesity and pesticide exposure are (among others) risk factors
153 for impaired postnatal metabolism and the occurrence of obesity or type 2 diabetes in
154 the offspring. Furthermore, neonatal parathion exposure of rats on an HFD favoured
155 the incidence of obesity independently of dietary factors (Adigun et al., 2010). Lastly,

156 it has been reported that an obese phenotype can exacerbate the toxicity of CPF
157 exposure in mice (Kondakala et al., 2017). Hence, the primary objective of the
158 present study was to determine whether, in rats, exposure of dams to CPF and an
159 HF diet, from 4 months before gestation to weaning, increased the occurrence of
160 obesity, metabolic disorders and liver damage among their male offspring.

161 **2. Methods**

162

163 2.1. Animal Housing

164

165 We studied 51 male offspring of 16 female Wistar rats (Janvier laboratory - Saint
166 Berthevin, France. All animals were pair-housed in stainless-steel, enriched cages
167 (with a roller, a sizzle pad, and hardwood chip bedding) at a controlled temperature
168 ($23\pm 1^\circ\text{C}$), at constant relative humidity (26%) with a 12-hour/12-hour dark/artificial
169 light cycle (El Khayat El Sabbouri et al., 2020).

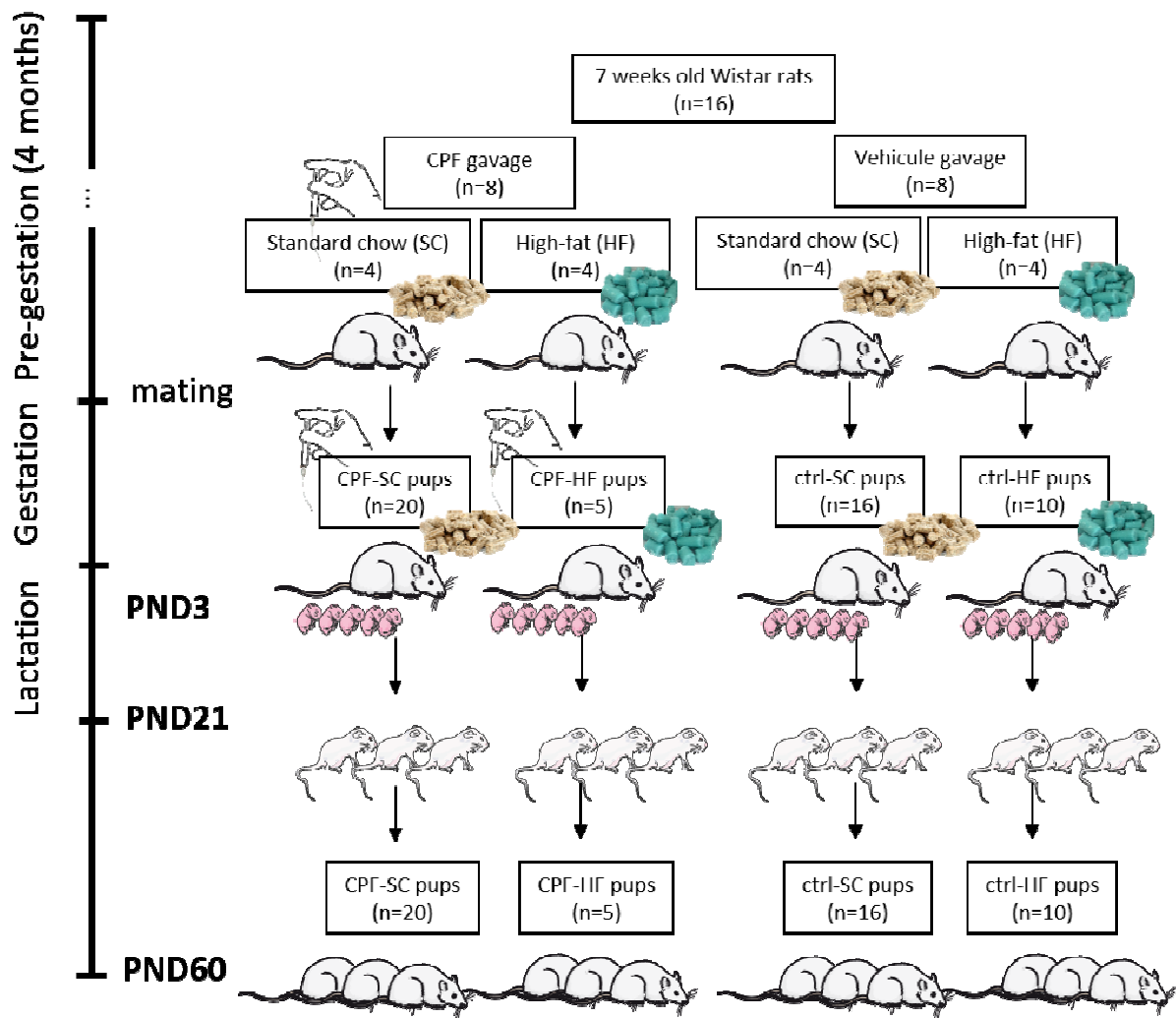
170 The animals' welfare was monitored daily. All experimental protocols had been
171 audited by the local animal care and use committee (C2EA#96, Amiens, France) and
172 approved by the French Ministry of Research (reference: APAFIS # 8207-
173 2016121322563594 v2). All animal experiments complied with the European Union
174 Directive 2010/63/EU.

175

176 2.2. Animal Experiments

177

178 The male offspring of dams exposed to the different environmental conditions were
179 monitored from birth until early adulthood, i.e. postnatal day (PND) 60. The offspring
180 were not exposed to chemical treatments or non-standard environmental factors and
181 had *ad libitum* access to a standard chow diet (SCD, Serlab 3436 maintenance
182 chow, Serlab, Montataire, France) and water (Figure 1) throughout the study. After
183 one week of acclimation, the 16 dams (mean \pm standard error of the mean body
184 weight: 225 ± 17 g at 8 weeks of age) were assigned at random to the two
185 experimental groups: one group (n=8) received chlorpyrifos-ethyl (CPF, O,O-diethyl
186 O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate (LGC Standards Laboratory,
187 Molsheim, France), 1 mg/kgBW/d *per os* in 1 mL/kg rapeseed oil vehicle (MP
188 Biomedicals, Illkirch, France)) and the other group (n=8) received vehicle alone (1
189 mL/kg) (Figure 1).



190

191 **Figure 1 – The experimental plan.**

192 The left-hand panel shows the experiment's timeline, and the main panel shows the
 193 groups and events for the dams and the offspring. CPF, chlorpyrifos; HF, high-fat
 194 diet; SC, standard chow diet; PND, postnatal day.

195

196 The CPF and non-CPF groups were each subdivided into an HF group (n=4, in which
 197 60% of the energy came from fat; D12492 chow; Ssniff Spezialdiäten GmbH, Soest,
 198 Germany) or an SCD group (n=4, with Serlab 3436 maintenance chow or, during
 199 gestation and lactation, Serlab 3336 breeding chow) (Figure 1). This model has been
 200 shown to induce metabolic alterations (elevated BW, hyperinsulinemia, programming
 201 of adiposity, etc.) in the offspring at adulthood (7 months of age) (Lecoutre et al.,
 202 2016). A recent study showed that standard maintenance chow (as used in the
 203 present experiment) is a suitable control for a 16- to 18-week HFD study; a low-fat

204 diet is not necessarily required for effects on the phenotype (weight gain),
 205 metabolism (glucose tolerance, levels of high-density lipoprotein (HDL), triglycerides
 206 (TGs), plasma cytokines and adipocytokines) and behavioural outcomes (Almeida-
 207 Suhett et al., 2019).

208 Furthermore, we studied the HFD's transgenerational influence on the offspring (all of
 209 which received a SC diet), rather than on the exposed dams. Further details of the
 210 diets are given in Table 1.

211

212 **Table 1- Composition of the rodent chows used in the study.**

213 a. Contains (in g/kg, totalling 1000): pork lard (315), casein (277), maltodextrin (156),
 214 sucrose (85), cellulose powder (60), mineral premix (60), soybean oil (31), vitamin
 215 premix (10), L-cystine (3.5), choline chloride (2.5), dye (blue)(0.3), butylated
 216 hydroxytoluene (0.1), corn starch (0).

217 Fatty acid composition of the HFD: lauric acid (0.07%), myristic acid (0.44%), palmitic
 218 acid (7.93%), stearic acid (4.37%), arachidic acid (0.11%), linolenic acid (0.49%),
 219 linoleic acid (4.64%), palmitoleic acid (0.94%), and oleic acid (13.97%)

220 b. Contains: wheat middling, wheat, barley, soybean meal (non-GMO), corn (non-
 221 GMO), poultry meal, wheat starch, whey powder, soybean oil (2.13%), sunflower
 222 cake, brewer's dried yeast, minerals, vitamins, and amino acids

223 c. Contains: flaked oats, soybean meal (non-GMO), poultry meal, wheat middling,
 224 wheat starch, barley, herring meal, grass meal, whey powder, corn (non-GMO),
 225 soybean oil, minerals, vitamins, and amino acids

226

Crude nutrients (%)	HF diet^a	SC diet	Standard breeding chow diet^c
Product reference	D12492	3436	3336
Supplier	Ssniff Spezialdiäten GmbH, Soest, Germany	Serlab, Montataire, France	Serlab, Montataire, France

Purpose	High-calorie diet	Maintenance diet	Pregnancy/ lactation
Gross energy (GE) MJ/kg	25	16.1	17
Metabolizable energy (ME) MJ/kg	21.6	13.1	14.3
Metabolizable energy density (Kcal/g)	5.16	3.13	3.41
Major nutrients (%)			
Crude protein	24.4	18.5	23.5
Crude fat	34.6	4.5	5.5
Crude fibre	6.0	4.5	3.0
Crude ash	5.3	6.5	5.7
Starch	0.1	35	36
Sugar	9.4	Unknown	Unknown
N-free extracts	26.3	54	51.3
Minerals (%)			
Calcium	0.92	1.05	1.00
Phosphorus	0.64	0.80	0.74
Magnesium	0.23	0.20	0.20
Sodium	0.20	0.20	0.20

Potassium	0.97	0.78	0.80
Chlorine	Unknown	0.36	0.33
Amino acids (%)			
Arginine	0.95	1.10	1.50
Lysine	2.02	1.00	1.40
Methionine	0.86	0.39	0.60
Methionine + cysteine	1.31	0.76	0.60
Tryptophan	0.33	0.20	0.27
Threonine	1.07	0.65	0.87
Vitamins (/kg)			
Vitamin A	15,000 IU	14,000 IU	14,000 IU
Vitamin D3	1,500 IU	1,000 IU	1,000 IU
Vitamin E	150 mg	110 mg	110
Vitamin K	20 mg	2 mg	2 mg
Thiamine (B1)	25 mg	30 mg	32 mg
Riboflavin (B2)	16 mg	20 mg	21 mg
Pyridoxine (B6)	16 mg	14 mg	14 mg
Cobalamin (B12)	30 µg	50 µg	50 µg
Nicotinic acid	47 mg	70 mg	70 mg
Pantothenic acid	55 mg	33 mg	33 mg

Folic acid	16 mg	2 mg	2.4 mg
Biotin	300 µg	220 µg	220 µg
Choline	920 mg	2000 mg	2000 mg
Vitamin C	Unknown	40 mg	40 mg
Trace elements (mg/kg)			
Iron	168	250	200
Zinc	65	60	68
Copper	13	14	15
Iodine	1.2	1	1
Manganese	95	60	40
Selenium	0.2	0.3	0.27

227

228 At the age of 24 weeks (i.e. after 4 months of exposure), the 16 dams (mean body
229 weight: 316 ± 33 g BW) were mated. Pregnancy was confirmed by the presence of
230 spermatozoa and/or a plug in the vagina, checked twice with a smear early on the
231 following morning. After fertilization, pregnant females were individually housed in
232 clean plastic cages. The various treatments and diets (CPF vs. vehicle, and HF diet
233 vs. SC diet) were continued until the end of the lactation period. PND1 was defined
234 as the day of parturition. On average, a litter comprised 10 ± 5 pups, with 4 ± 3
235 males. There were no intergroup differences in the litter size. Male pups accounted
236 for 54% of the offspring. Each pup was tattooed on the leg with a special veterinary
237 ink (Centravet, Nancy, France) for individual identification. The pups were counted,
238 sexed and weighed at PND3. To limit potential maternal separation stress in early
239 life, the pups were not weighed at PND1. At weaning (PND21), the pups were
240 separated from their mothers and were fed tap water and standard chow (Serlab
241 3436) *ad libitum* (Figure 1). In order to avoid potentially confounding factors (such as
242 menstrual cycles and the impact of hormones on physiological functions), only male

243 pups were studied further (Figure 1) in the following four groups: (i) the ctrl-SCD
244 group (maternal exposure to SCD and vehicle, n=16); (ii) the CPF-SCD group
245 (maternal exposure to SCD and CPF, n=20), (iii) the ctrl-HFD group (maternal
246 exposure to an HFD and vehicle, n=10), and (iv) the CPF-HFD group: maternal
247 exposure to both CPF and HFD (n=5).

248 Due to poor fertility, the number of male pups was lower in the co-exposure group
249 (CPF-HF) than in the other groups. Pups were weighed every 3 days until PND54
250 (early adulthood). Animals were fasted on the night before sacrifice. Rats were
251 euthanized at PND60 (8 weeks of age) with a single, lethal, intraperitoneal injection
252 of pentobarbital sodium (EXAGON®, Axience SAS, Pantin, France; 140 mg/kg - 0.35
253 mL/kg). Tissue samples and blood were immediately harvested under sterile
254 conditions and stored at -80°C. For the gene expression study, liver tissue samples
255 were harvested and dived into the Invitrogen RNA/*later* Stabilization Solution (Life
256 Technologies, Thermo Fisher Scientific, Courtaboeuf, France) - a non-toxic tissue
257 storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA.
258 Plasma samples were obtained by centrifugation and immediately frozen at -80°C.

259

260 2.3. Evaluation of possible impairment of lipid and glucose metabolism in
261 offspring exposed perinatally to CPF and/or an HF diet

262

263 2.3.1. Gene expression of proteins involved in lipid and carbohydrate
264 metabolism, as measured in a reverse transcription polymerase chain
265 reaction (RT-PCR)

266

267 Total RNA was isolated from liver samples using the FastGene Basic ® RNA kit
268 (NIPPON Genetics Europe, Dueren, Germany). The total RNA concentration was
269 measured spectrophotometrically using a Nanodrop 1000 spectrophotometer (Life
270 Technologies). cDNA was generated by reverse transcription of 1 µg mRNA with the
271 High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life
272 Technologies), as described previously (Joly Condet et al., 2015). The expression
273 of genes coding for proteins involved in lipid and carbohydrate metabolism was
274 measured using RT-PCRs (Table 2).

275

		Gene name	Forward primer	Reverse primer	T m	Accession number and reference
HOUSEKEEPING GENES	<i>Gapdh</i>	Glyceraldehyde 3 phosphate dehydrogenase	AGACAGCC GCATCTTCT TGT	CTTGCCGT GGGTAGA GTCAT	60	<u>NM 01700</u> <u>8.4</u> (Melgar-Rojas et al., 2015)
	<i>Actb</i>	Beta actin	ACGTCGAC ATCCGCAA AGACCTC	TGATCTCC TTCTGCAT CCGGTCA	60	(Li et al., 2018)
	<i>Hprt1</i>	Hypoxanthine guanine phosphoribosyl transferase 1	TCGTACCTT TCTCACCAC AGTATCTAG	GAAAACATA AGACACCT CCCCATCA	60	<u>NM 01731</u> <u>4.1</u> (Long et al., 2019)
	<i>Ubc</i>	Ubiquitin C	CTCATGGA CTGATTATG GACAGGAC	GCAGGTC AGCAAAG AACTTATA GCC	60	<u>NM 01258</u> <u>3</u> (Hermier et al., 2017)
LIPOGENESIS	<i>Srebf1</i>	Sterol regulatory element binding protein 1 c	GCTGTTGG CATCCTGCT ATC	TAGCTGG AAGTGAC GGTGGT	60	<u>NM 01148</u> <u>0</u> (Li et al., 2018)
	<i>Acaca</i>	Acetyl-CoA carboxylase 1	TGTCTATTC GGGGTGAC TTTCG	CCGTTCAT TATCACCA CGTAGG	60	<u>NM 02219</u> <u>3</u> (Ahmed et al., 2015)
	<i>Fasn</i>	Fatty acid	GAGCTGCG	GCCGCCG	60	<u>NM 017332</u>

		synthase	GCTACGTG GCTA	TGAGGTT GCTGTT		(Ahmed et al., 2015)
	<i>Lpl</i>	Lipoprotein lipase	CAAGATTCA CTTTTCTGG GACTGA	GCCACTG TGCCGTA CAGAGA	60	<u>NM 01259</u> <u>8.2</u> (Mukherjee et al., 2008)
	<i>Mlxipl</i>	Carbohydrate response element binding protein	ACAACCCC TGCCTTACA CAG	GAGGTGG CCTAGGT GGTGTA	60	<u>NM 13355</u> <u>2.1</u> (Vida et al., 2014)
ENERGY METABOLISM	<i>Prkaa1</i>	AMP-activated protein kinase α	ATTTGCCCA GTTACCTCT TTCC	GCTTGTT CATTATTC TCCGAT	60	<u>NM 02399</u> <u>1.1</u> (Li et al., 2018)
GLUCONEOGENESIS	<i>G6pc</i>	Glucose-6-phosphatase	CGTGGAAA GAAAAAGT CAAC	GTAAAATC CAAGTGC GAAAC	60	<u>NM 01309</u> <u>8</u> (Seo et al., 2010)
	<i>Slc2a2</i>	Glucose transporter 2	AAGGATCA AAGCCATG TTGG	GGAGACC TTCTGCTC AGTGG	60	<u>NM 01287</u> <u>9</u> (Ahmed et al., 2015)
	<i>Pck1</i>	Phosphoenolpyruvate carboxykinase	GAAAGTTG AATGTGTG GGTGAT	TTCTGGGT TGATGGC CCTTA	60	<u>NM 19878</u> <u>0</u> (Stepien et al., 2011)

CHOLESTEROL SYNTHESIS	<i>Hmgcr</i>	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase	ACTATGGTT CTTTCCGTG CC	AAAACACC AGCTTCCA GCTT	60	<u>NM 01313</u> <u>4</u> (Seo et al., 2010)
FA OXIDATION	<i>Ppara</i>	Peroxisome proliferator activated receptor alpha	ATTGGCGTT CGCAGCTG TTT	CTCGTGT GCCCTCC CTCAAG	60	<u>NM 01319</u> <u>6</u> (Zhang et al., 2018)
LIPID ACCUMULATION	<i>Cd36</i>	Cluster of differentiation 36 also known as fatty acid translocase	AACCCAGA GGAAGTGG CAAAG	GACAGTG AAGGCTC AAAGATG G	60	<u>NM 03156</u> <u>1.2</u> (Chen et al., 2016)
	<i>Pparg</i>	Peroxisome proliferator-activated receptor gamma	GCTGGCCT CCCTGATG AATA	GCTTCCG CAGGCTTT TGA	60	<u>XM 03290</u> <u>5882.1</u> (Sloop et al., 2004)
	<i>Dgat1</i>	Diacylglycerol O-acyltransferase 1	GGACAAAG ACCGGCAG ACCA	CAGCATCA CCACGCA CCAAT	60	<u>NM 05343</u> <u>7</u> (Chen et al., 2016)
	<i>Dgat2</i>	Diacylglycerol O-acyltransferase 2	CCTGGCAA GAACGCAG TCAC	GAGCCCT CCTCAAAG ATCACC	60	<u>NM 00101</u> <u>2345</u> (Chen et al., 2016)
FIBROSIS	<i>Col1a1</i>	Collagen type I alpha 1 chain	TACAGCAC GCTTGTGG ATGG	CAGATTG GGATGGA GGGAGTT	60	<u>NM 05330</u> <u>4.1</u> (Seo et al., 2010)

INFLAMMATION	<i>IL1b</i>	Interleukin 1 beta	CTTCCTTGT GCAAGTGT CTGAAGC	AAGAAGG TCCTTGG GTCCTCAT CC	60	<u>NM 03151</u> <u>2.2</u> (Shih et al., 2005)
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278

279 Primers and SYBR™ Green Master Mix were purchased from Life Technologies.
280 PCRs were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems,
281 Foster City, CA, USA). Reactions were performed in duplicate.

282 Four housekeeping genes (*Gapdh*, *Hprt1*, *Ubc*, and *Actb*) were tested under the
283 same conditions. Expression stability was determined using RefFinder (Xie et al.,
284 2012) BestKeeper (Pfaffl et al., 2004), Normfinder (Andersen et al., 2004) and
285 Genorm (Vandesompele et al., 2002) programs with the help of the delta-Ct method
286 (Silver et al., 2006). After a comparison of the results, *Gapdh* was selected as the
287 best housekeeping gene (Supplementary Material 1). Levels of amplified cDNA were
288 quantified using the $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct = \Delta Ct_{\text{exposed}} - \text{mean } \Delta Ct_{\text{control}}$), relative to
289 *Gapdh*.

290

291 2.3.2. Assays of lipid and carbohydrate metabolic markers in plasma

292

293 Plasma glucose and lipids (TGs, total cholesterol (CT), and HDL) were assayed on a
294 Roche COBAS analyser (Roche Diagnostic, Meylan, France). The concentrations of
295 very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) were calculated
296 as $VLDL = TG/2.2$ and $LDL = CT - HDL - VLDL$. Liver enzymes: alkaline
297 phosphatase (ALP), aspartate aminotransferase (ASAT), alanine aminotransferase
298 (ALAT), and lactate dehydrogenase (LDH), were also assayed on a Roche COBAS
299 analyser. Lastly, leptin and insulin were assayed using the specific rat ELISA Kit (#
300 KRC2281 and #ERINS; Life Technologies) with detection ranges of 62.5-4000 pg/mL
301 and 4.69-300 $\mu\text{IU/mL}$ respectively. All plasma assays were performed in the
302 Endocrinology Laboratory at Cochin Hospital (Paris, France).

303

304 2.4. Evaluation of impairments in hepatic metabolism in offspring exposed
305 perinatally to CPF and/or an HF diet

306

307 2.4.1. Hepatic TG levels

308

309 Hepatic TG levels were evaluated in 10 mg of liver tissue, using the PicoProbe
310 Triglyceride Quantification Assay Kit (Fluorometric) ab178780 from Abcam,
311 Cambridge, United Kingdom), according to the manufacturer's instructions. Liver TG
312 levels (expressed in mg/g liver tissue) were determined in a microplate-based
313 fluorometric assay (excitation/emission = 535/587 nm). In order to relate the well
314 fluorescence intensity to the TG level, a standard curve (from 0 to 4000 pmol/mL)
315 was generated. All assays were performed in duplicate.

316

317 2.4.2. Liver tissue structure

318

319 Immediately after sacrifice, pieces of liver left lobe were fixed in Carnoy's solution
320 (60% absolute alcohol, 30% chloroform, and 10% acetic acid v/v) during 48 h before
321 being embedded into xylene and then paraffin. Samples were then sliced into 5 µm
322 sections and transferred to glass slides for staining with three different reagents. The
323 first set of slides was stained with periodic acid Schiff (PAS) reagent (395B-1KT,
324 Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), in order to (i) highlight
325 carbohydrates (mucins, basal *lamina*, glycogen...) in the liver tissue, and (ii) estimate
326 liver glycogen levels (based on the proportion of PAS-positive hepatocytes). The
327 second set of slides was stained with Picro Sirius Red, in order to visualize collagen
328 type I and III fibres and evaluate fibrosis (if present). The third set of slides was
329 treated with Masson's trichrome stain, to reveal collagen fibres. Histological sections
330 were observed under the light microscope (Olympus U TV1 X, Olympus, Japan) by a
331 histopathologist who was blinded to the group assignment. Photos were taken using
332 a camera attached to the microscope (Olympus DP-10 Digital Camera, Olympus
333 America, New York). Staining was quantified on two fields/slide (100x magnification)
334 with the help of ImageJ software (Schneider et al., 2012).

335

336 2.5. Statistical analysis

337

338 Data were expressed as the mean \pm standard error of the mean (SEM) and were
339 analysed using GraphPad Prism software (version 5.04 for Windows, GraphPad
340 Software Inc., San Diego, CA, USA www.graphpad.com). Groups were compared
341 using a non-parametric Kruskal-Wallis test; if the intergroup difference was
342 statistically significant, Dunn's post-hoc test was then applied. The threshold for
343 statistical significance was set to $p < 0.05$.

344

345 For additional information, please refer to the Key Resources Table.

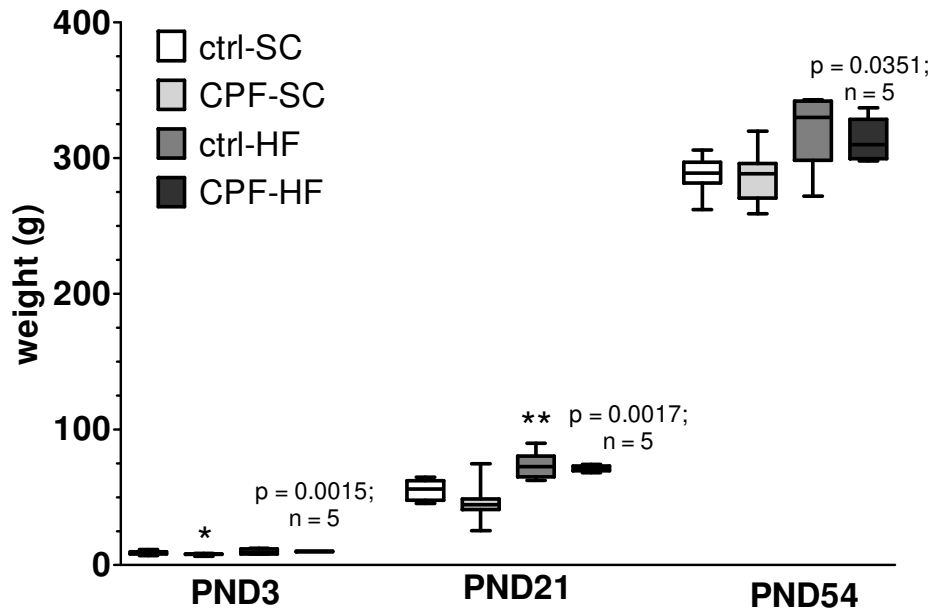
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3. Results

3.1. Perinatal exposure to CPF with an SC or an HF diet is associated with alterations in the growth rate and glycaemia but not in lipemia

3.1.1. Perinatal exposure to CPF is associated with slower pup growth from birth until weaning

Male pups from mothers exposed to CPF had a significantly lower mean \pm SEM body weight immediately after birth (PND3; 7.99 g \pm 0.11 g in the CPF-SC group, vs. 9.04 g \pm 0.32 g in the ctrl-SC group; relative decrease: 12%; p=0.0196) (Figure 2). In contrast, perinatal exposure to an HF diet was associated with a significantly greater body weight at weaning (PND21: 73.3 g \pm 3.03 g in the ctrl-HF group, vs. 55.5 g \pm 1.90 g in the ctrl-SC group; relative increase: 32%; p=0.0317). At the end of the study (PND54), the body weight was greater (but not significantly) in the ctrl-HF group (322.2 g \pm 12.99 g) than in the ctrl-SC group (288.8 g \pm 4.36 g; relative increase: 12%; p=0.1932). Furthermore, perinatal HF diet exposure was associated with a significantly greater body weight in CPF-HF rats soon after birth (PND3; 10.09 g \pm 0.13 g, vs. 7.99 g \pm 0.11 g in CPF-SC; relative increase: 26%; p=0.0015), at weaning (PND21; 71.27 g \pm 0.99 g in CPF-HF vs. 47.20 g \pm 2.60 g in CPF-SC; relative increase: 51%; p=0.0017) and at early adulthood (PND54; 313.2 g \pm 7.08 g in CPF-HF vs. 285.0 g \pm 3.64 g in CPF-SC; relative increase: 10%; p=0.0351) (Figure 2).



368

369 **Figure 2** –Body weight (g) at birth (PND3), after weaning (PND21) and in early
 370 adulthood (PND54) in male offspring born to mothers exposed to CPF and/or an HF
 371 diet.

372 Data were expressed as the mean \pm SEM (n=5-20). * ** significant difference
 373 (p<0.05 and p<0.01, respectively) vs. ctrl-SC offspring. ctrl, control; CPF;
 374 chlorpyrifos; HF, high-fat diet; SC, standard chow diet; PND; postnatal day.

375

376 3.1.2. Perinatal CPF exposure alters regulation of the energy balance

377

378 The hepatic and plasma TG levels were higher (by 14% and 20%, respectively) in
 379 male offspring of dams from the CPF-SC group, relative to the ctrl-SC offspring.
 380 However, these differences were not statistically significant. Perinatal HFD exposure
 381 was also associated with a higher hepatic TG content (by 35%, vs. the ctrl-SC
 382 offspring: p=NS). There were no significant intergroup differences in the lipid profile
 383 (Table 3).

384 Plasma levels of insulin-like growth factor-1 (IGF1) and leptin were significantly lower
 385 in the CPF-SC offspring than in the ctrl-SC offspring (relative decreases: 49.11%,
 386 p=0.0043, and 57.80%, p=0.0321, respectively). The insulin level and glycaemia
 387 were slightly but not significantly higher (relative increases: 20% and 23%,
 388 respectively) in the CPF-SC offspring than in the ctrl-SC offspring. Perinatal HFD

389 exposure was associated with a slightly but not significantly higher glycaemia value
 390 (relative increase vs. ctrl-SC: 16%). However, there were no significant differences
 391 between the CPF-HF and ctrl-HF groups with regard to glycaemia or hormone levels
 392 (Table 3).

393

394 **Table 3 - Lipid and carbohydrate metabolism in 8-week-old male offspring of**
 395 **dams exposed to CPF and/or an HFD.**

396 Plasma samples were assayed for total cholesterol (TC), plasma and hepatic
 397 triglycerides (TGs), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and
 398 glycaemia-associated hormones. The data were quoted as the mean \pm SEM (n=5-
 399 20). *,** significant difference ($p < 0.05$ and $p < 0.01$, respectively) vs. ctrl-SC offspring.
 400 ctrl, control; CPF; chlorpyrifos; HF, high-fat diet; SC, standard chow diet; IGF1,
 401 insulin-like growth factor-1.

402

	ctrl-SC	CPF-SC	ctrl-HF	CPF-HF
TC ($\mu\text{mol/L}$)	2214 \pm 72.6	2252 \pm 92.0	2064 \pm 86.4	1987 \pm 63.3
TG ($\mu\text{mol/L}$)	951 \pm 73.7	1139 \pm 103.1	960 \pm 94.4	1463 \pm 69.8
TG (mg/g liver)	24.21 \pm 3.5	27.37 \pm 1.4	32.89 \pm 4.0	36.06 \pm 8.21
HDL ($\mu\text{mol/L}$)	1533 \pm 62.1	1573 \pm 74.1	1470 \pm 69.7	1323 \pm 57.8
LDL ($\mu\text{mol/L}$)	478.3 \pm 32.3	426.6 \pm 25.2	759.7 \pm 25.7	370.7 \pm 15.8
Fasting glycaemia (mmol/L)	11.69 \pm 0.8	14.43 \pm 0.8	13.53 \pm 1.3	14.99 \pm 2.4
Fasting leptin (pg/mL)	622.3 \pm 86.3	305.6 \pm 33.6 *	759.7 \pm 163.1	469.1 \pm 63.9
Fasting insulin ($\mu\text{U/L}$)	10.12 \pm 0.9	12.19 \pm 1.3	9.73 \pm 0.7	10.33 \pm 3.8

Fasting IGF1 (ng/mL)	6.47 ± 0.33	3.74 ± 0.57 **	6.00 ± 0.50	4.61 ± 1.73
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403

404 3.2. Perinatal exposure to CPF (but not to an HFD) is associated with
405 significantly lower expression of genes coding for lipid metabolism

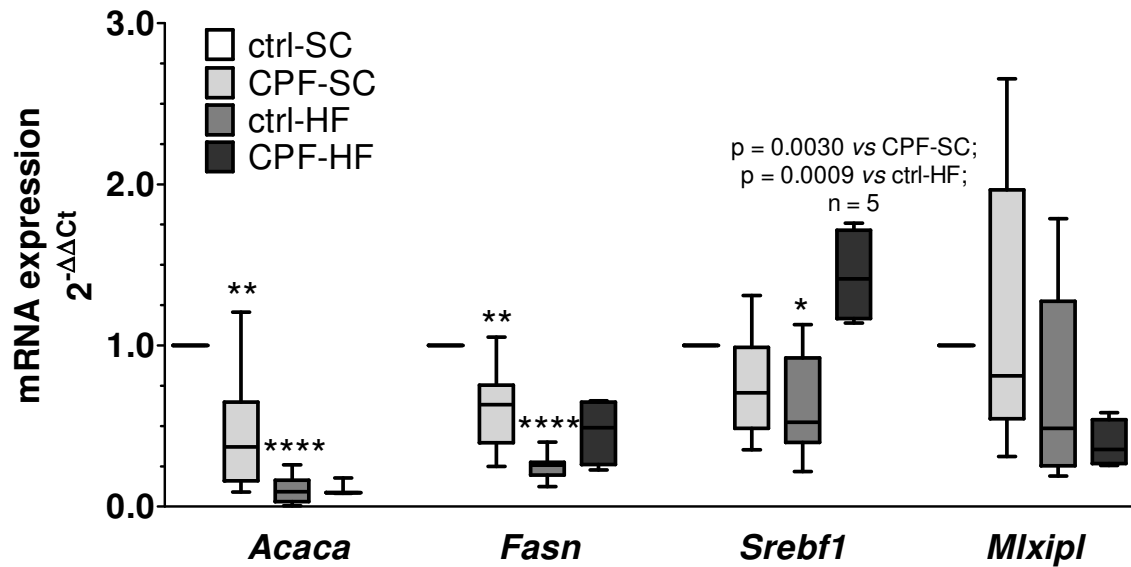
406

407 Perinatal CPF exposure was associated with significantly lower mRNA expression of
408 acetyl-CoA carboxylase 1 (*Acaca*; relative decrease vs. ctrl-SC: 58%; p=0.0019) and
409 fatty acid synthase (*Fasn*, responsible for the synthesis of malonyl-CoA from acetyl
410 CoA) (relative decrease: 39%; p=0.0023). Exposure to both CPF and an HFD was
411 associated with significantly greater expression of *Srebf1/Srebp1c* (coding for sterol
412 regulatory element binding protein, a central regulator of lipogenesis) than for
413 exposure to CPF alone (relative increase: 230%; p=0.0009 vs. CPF-SC; Figure 3).

414 Furthermore, perinatal HFD exposure was associated with significantly greater
415 expression of *Acaca* (relative increase vs. ctrl-SC: 90%; p<0.0001); *Fasn* (relative
416 increase: 75%; p<0.0001) and *Srebf1/Srebp1c* (relative increase: 37%; p=0.0340).

417 Exposure to both CPF and an HFD was associated with significantly lower
418 expression of *Srebf1/Srebp1c* than for exposure to CPF alone (relative decrease vs.
419 CPF-SC: 197%; p=0.0030). However, we did not observe any significant intergroup
420 differences in the expression of *Mlxipl/Chrebp* (carbohydrate response element
421 binding protein, another major transcription factor involved in lipid synthesis) (Figure
422 3).

423



424

425 **Figure 3** – mRNA expression (RT-PCR) of lipogenesis markers in 8-week-old male
 426 offspring of dams exposed to CPF and/or an HF diet.

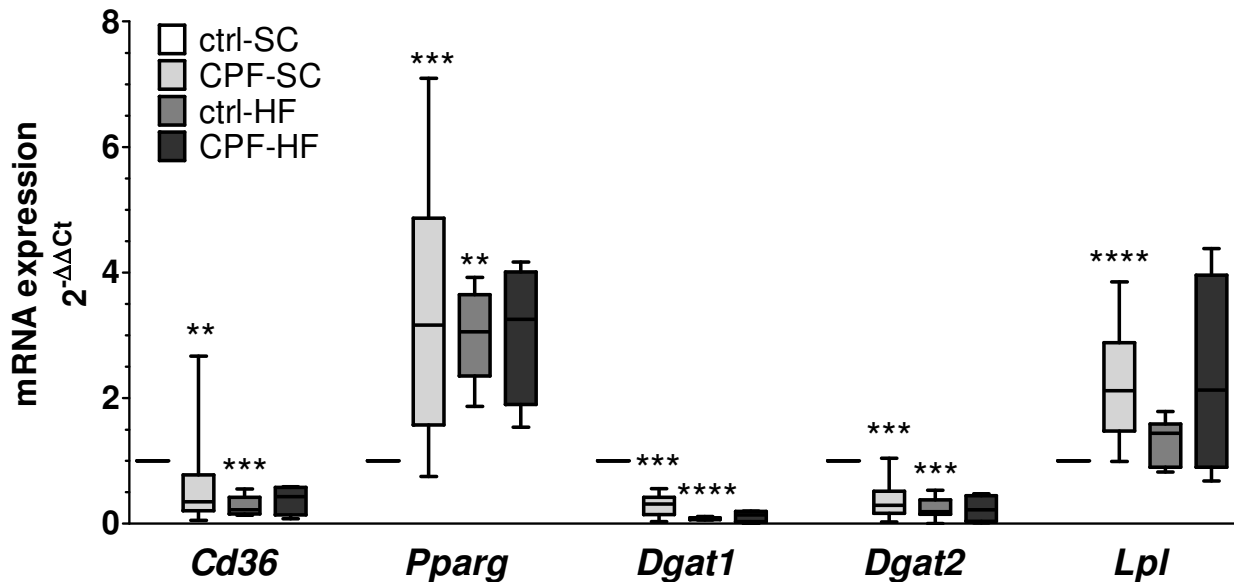
427 The mRNA gene expression levels were quantified as the fold induction over the
 428 control group (set to 1). Data were expressed as the mean \pm SEM (n=5-20), using the
 429 $2^{-\Delta\Delta C_t}$ calculation method with the *Gapdh* housekeeping gene. *, ** and *** statistically
 430 significant difference (p<0.05, p<0.01, and p<0.001, respectively) vs. ctrl-SCD
 431 offspring. ctrl, control; CPF; chlorpyrifos; HF, high-fat diet; SC, standard chow diet;
 432 *Acaca*, acetyl-CoA carboxylase 1; *Fasn*, fatty acid synthase; *Mlxipl*, carbohydrate
 433 response element binding protein; *Srebf1*, sterol regulatory element binding factor 1
 434 variant 2.

435

436 Perinatal CPF exposure was associated with significantly greater mRNA expression
 437 of the *Pparg* gene (coding for peroxisome proliferator-activated receptor gamma, a
 438 nuclear receptor involved in lipid storage; relative increase in the CPF-SC group vs.
 439 ctrl-SC: 238%; p=0.0002) and the lipoprotein lipase (*Lpl*) gene (relative increase:
 440 126%; p<0.0001). This exposure was associated with significantly lower mRNA
 441 expression of the fatty acid transporter *Cd36* (relative decrease vs. ctrl-SC: 38%,
 442 p=0.0015) and the diacylglycerol O-acyltransferases *Dgat1* and *Dgat2* involved in
 443 lipid transport (relative decreases vs. ctrl-SC: 70% and 65%, respectively; p=0.0005
 444 and p=0.0002, respectively).

445 Perinatal HFD exposure was associated with significantly greater mRNA expression
 446 of the nuclear receptor *Pparg* (relative increase vs. ctrl-SC: 238%; p=0.0033). In

447 contrast, this exposure was associated significantly lower mRNA expression of *Cd36*
 448 (relative decrease vs. ctrl-SC: 73%; p=0.0003), *Dgat1* (relative decrease: 92%;
 449 p<0.0001) and *Dgat2* (relative decrease: 76%; p=0.0002) (Figure 4).



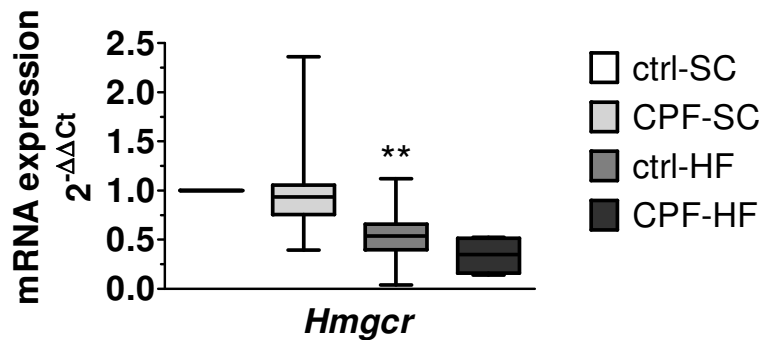
450

451 **Figure 4** – mRNA expression (RT-PCR) of markers of lipid accumulation in 8-week-
 452 old male offspring of dams exposed to CPF and/or an HF diet.

453 The mRNA gene expression levels were quantified as the fold induction, relative to
 454 the control group (set to 1). Data were expressed as the mean ± SEM (n=5-20), using
 455 the 2^{-ΔΔCt} calculation method with the *Gapdh* housekeeping gene. **, *** and ****
 456 statistically significant difference (p<0.01, p<0.001 and p<0.0001, respectively) vs.
 457 ctrl-SC offspring. ctrl, control; CPF; chlorpyrifos; HF, high-fat diet; SC, standard chow
 458 diet; *Cd36*; fatty acid translocase; *Dgat1*; Diacylglycerol O-acyltransferase 1; *Dgat2*;
 459 Diacylglycerol O-acyltransferase 2; *Lpl*; lipoprotein lipase; *Pparg*; peroxisome
 460 proliferator-activated receptor gamma.

461

462 Perinatal exposure to an HF diet was associated with significantly lower mRNA
 463 expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*Hmgcr*, a major
 464 involved in lipid (cholesterol) synthesis), relative to the ctrl-SC offspring (relative
 465 decrease: 39%; p=0.0069) (Figure 5).



466

467 **Figure 5** - mRNA expression (RT-PCR) of markers of cholesterol synthesis in 8-
 468 week-old male offspring of dams exposed to CPF and/or an HF diet.

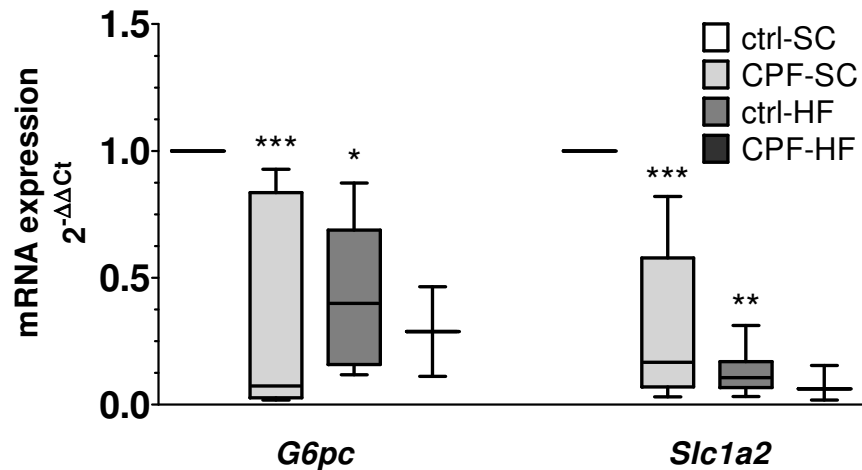
469 The mRNA gene expression levels were quantified as the fold induction, relative to
 470 the control group (set to 1). Data were expressed as the mean \pm SEM (n=5-20), using
 471 the $2^{-\Delta\Delta C_t}$ calculation method with the *Gapdh* housekeeping gene. ** statistically
 472 significant difference (p<0.01) vs. ctrl-SCD offspring. ctrl, control; CPF; chlorpyrifos;
 473 HF, high-fat diet; SC, standard chow diet; *Hmgcr*, 3-hydroxy-3-methyl-glutaryl-
 474 coenzyme A reductase.

475

476 3.3. Perinatal exposure to CPF is associated with significantly lower expression
 477 of genes coding for enzymes in glucose metabolism but this influence is
 478 not exacerbated by perinatal exposure to HF diet

479

480 Perinatal CPF exposure was associated with significantly lower mRNA expression of
 481 *G6pc* (coding for glucose-6-phosphatase; relative decrease vs. ctrl-SC: 60%;
 482 p=0.0002) and *Slc2a2/Glut2* (coding for a hepatic glucose transporter; relative
 483 decrease: 72%; p=0.0002). Perinatal HF diet exposure was associated with
 484 significantly lower mRNA expression of *G6pc* (relative decrease vs. ctrl-SC: 58%;
 485 p=0.0396) and *Slc2a2/Glut2* (relative decrease: 87%; p=0.0014) (Figure 6).



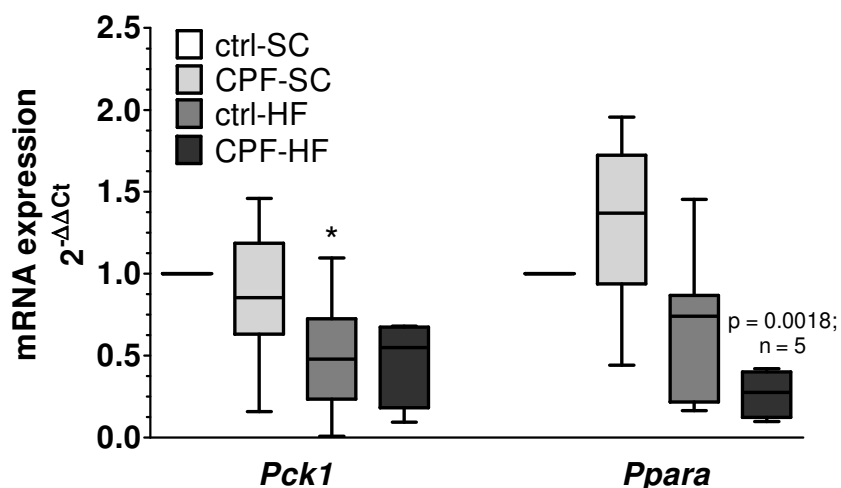
486

487 **Figure 6** - mRNA expression (RT-PCR) of markers of carbohydrate metabolism in 8-
 488 week-old male offspring of dams exposed to CPF and/or an HF diet.

489 The mRNA gene expression levels were quantified as the fold induction, relative to
 490 the control group (set to 1). Data were expressed as the mean \pm SEM (n=5-20), using
 491 the $2^{-\Delta\Delta C_t}$ calculation method with the *Gapdh* housekeeping gene. *, ** and ***
 492 statistically significant difference (p<0.05, p<0.01 and p<0.001, respectively) vs. ctrl-
 493 SCD offspring. CPF; chlorpyrifos; ctrl, control; HF, high-fat diet; SC, standard chow
 494 diet; *G6pc*, glucose-6-phosphatase; *Slc2a2*, solute carrier family 2, facilitated glucose
 495 transporter member 2.

496

497 Lastly, perinatal HFD exposure was associated with significantly lower mRNA
 498 expression of *Pck1/Pepck* (coding for phosphoenolpyruvate carboxykinase; relative
 499 decrease vs ctrl-SC: 50%; p=0.0207). Perinatal exposure to both HFD and CPF was
 500 associated with significantly lower mRNA expressed of *Ppara*, relative to CPF
 501 exposure alone (relative decrease vs. CPF-SC: 80%; p=0.0018) (Figure 7).



502

503 **Figure 7** - mRNA expression (RT-PCR) of markers of gluconeogenesis and fatty acid
 504 oxidation in 8-week-old male offspring of dams exposed to CPF and/or an HF diet.

505 The mRNA gene expression levels were quantified as the fold induction, relative to
 506 the control group (set to 1). Data were expressed as the mean ± SEM (n=5-20), using
 507 the $2^{-\Delta\Delta C_t}$ calculation method with the *Gapdh* housekeeping gene. * statistically
 508 significant difference (p<0.05) vs. ctrl-SCD offspring. ctrl, control; CPF; chlorpyrifos;
 509 HF; high-fat diet; SC, standard chow diet; *Pck1*, phosphoenolpyruvate
 510 carboxykinase; *Ppara*, peroxisome proliferator activated receptor alpha.

511

512 3.4. Perinatal exposure to an HF diet and/or CPF was not associated with
 513 changes in liver tissue structure

514

515 3.4.1. Perinatal exposure to CPF and/or an HF diet was not associated with
 516 histopathological changes in the offspring's liver

517

518 In all histopathological analyses, the offspring's liver tissues sections had a normal
 519 structure and composition (a central vein, and well-organized hepatocytes) (Figure 8
 520 A). Indeed, no histopathological changes (portal area hypertrophy, dilatation of bile
 521 duct or the branch of the hepatic artery, mononuclear cell infiltration, vacuolization,
 522 etc.) were observed in liver sections in any of the groups studied. PAS staining of
 523 liver sections showed that exposure of CPF and/or HFD was not associated with
 524 steatosis or inflammation (Figure 8A): the liver's tissue architecture (e.g. percentage
 525 of steatosis) was not modified, relative to ctrl-SCD offspring (data not shown).
 526 However, the hepatocyte surface area was significantly lower (by 17%; p=0.0145) in

527 the CPF-SCD offspring than in the ctrl-SCD offspring. No collagen fibres could be
528 seen between the hepatocytes – suggesting that the amount of collagen was below
529 the staining methods' limits of detection. Therefore, we did not observe any
530 characteristic features of hepatic fibrosis (Figure 8 B and C).

531

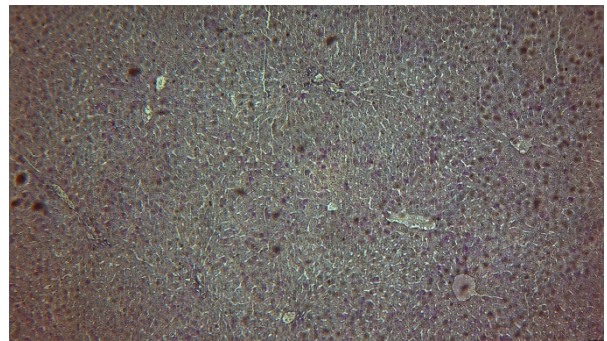
A

PAS staining

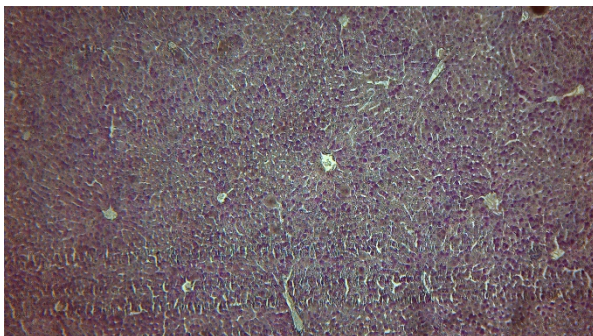
ctrl-SC



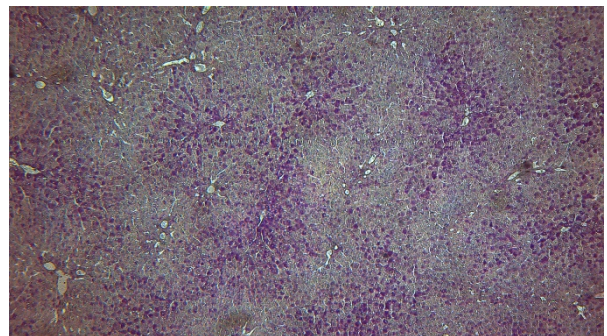
CPF-SC



ctrl-HF



CPF-HF



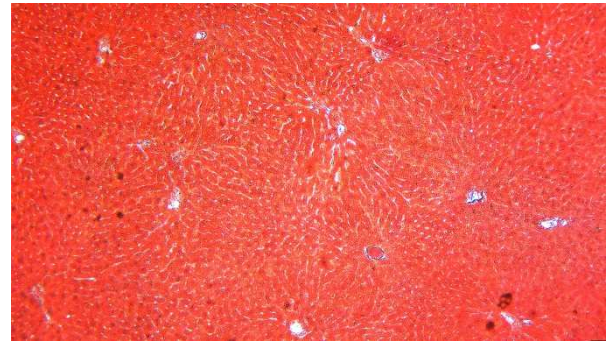
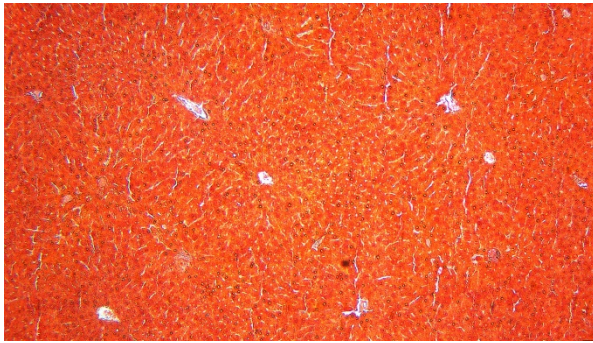
532

B

Masson's trichrome staining

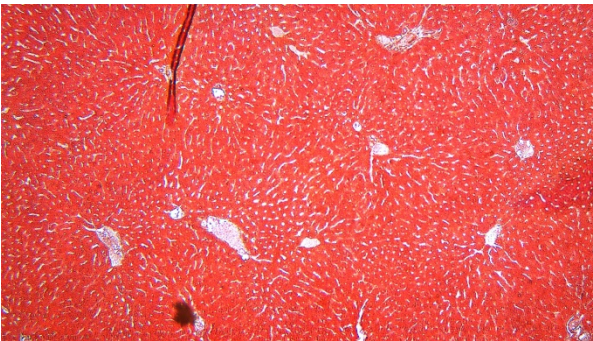
ctrl-SC

CPF-SC



ctrl-HF

CPF-HF

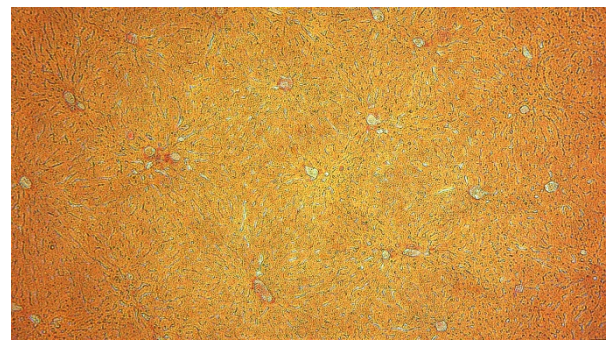
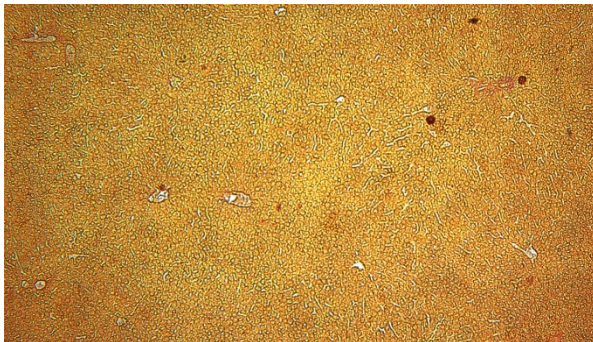


C

Sirius Red staining

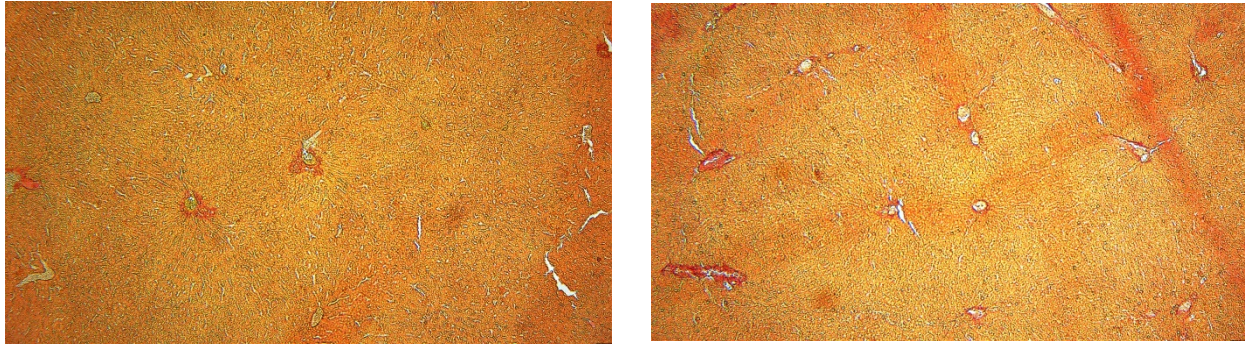
ctrl-SC

CPF-SC



ctrl-HF

CPF-HF



533

534 **Figure 8 – PAS, Masson's trichrome and Sirius Red staining of liver tissue in the 8-**
535 **week-old male offspring of mothers exposed to CPF and/or an HF diet.**

536

537 (A) PAS staining (magnification: 100x), (B) Masson trichrome staining of collagen
538 fibres (100x), (C) Sirius Red staining of collagen I and III fibres (100x). Data were
539 expressed as the mean \pm SEM (n=5-20): there were no significant intergroup
540 differences. ctrl, control; CPF; chlorpyrifos; HF, high-fat diet, SC, standard chow diet;
541 PAS; periodic acid Schiff.

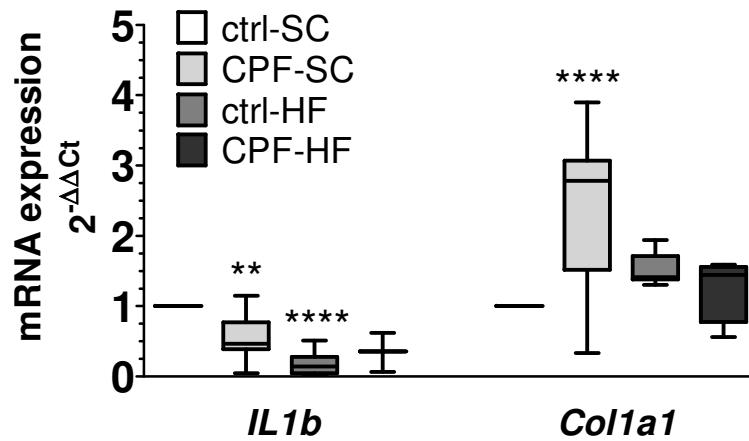
542

543 3.4.2. Perinatal CPF exposure might be associated with subsequent fibrosis

544

545 Perinatal exposure of dams to CPF was associated with significantly lower mRNA
546 expression of the *IL1b* gene (an inductor of the inflammatory response (relative
547 decrease vs. ctrl-SC: 46%; p=0.0071) and significantly greater mRNA expression of
548 *Col1a1* (coding for one of the two main collagen fibres; relative increase vs. ctrl-SC:
549 160%; p<0.0001) (Figure 9). Maternal exposure to an HFD was associated with
550 significantly lower *IL1b* gene expression (relative decrease vs. ctrl-SC: 82%;
551 p<0.0001) (Figure 9).

552



553

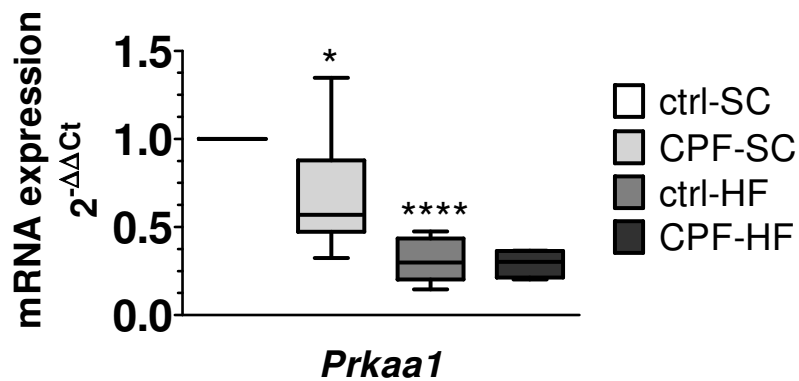
554 **Figure 9** - mRNA expression (RT-PCR) of markers of inflammation and fibrosis in 8-
 555 week-old male offspring of dams exposed to CPF and/or an HF diet.

556 The mRNA gene expression levels correspond to the fold-induction over the control
 557 group (set to 1). Data were expressed as the mean \pm SEM (n=5-20), using the $2^{-\Delta\Delta C_t}$
 558 calculation method with the *Gapdh* housekeeping gene. **, **** statistically significant
 559 difference (p<0.01 and p<0.0001, respectively) vs. ctrl-SC offspring. ctrl, control;
 560 CPF; chlorpyrifos; HF, high-fat diet; SC, standard chow diet; *Col1a1*; collagen alpha-
 561 1; *IL1b*; interleukin 1 beta.

562

563 The mRNA expression of *Prkaa1/Ampka* (coding for a sensor of the cell's energy
 564 homeostasis) was significantly lower (by 33%) in the CPF-SC offspring than in the
 565 ctrl-SC offspring (p=0.0448). Perinatal HFD exposure was associated with
 566 significantly lower mRNA expression of *Prkaa1/Ampka* (relative decrease vs. ctrl-SC:
 567 69%) (Figure 10).

568



570

571 **Figure 10** - mRNA expression (RT-PCR) of markers of energy metabolism in 8-
 572 week-old male offspring of dams exposed to CPF and/or an HF diet.

573 The mRNA gene expression levels were quantified as the fold induction, relative to
 574 the control group (set to 1). Data were expressed as the mean \pm SEM (n=5-20), using
 575 the $2^{-\Delta\Delta C_t}$ calculation method with the *Gapdh* housekeeping gene. *, **** statistically
 576 significant difference (p<0.05 and p<0.0001, respectively) vs. ctrl-SCD offspring. ctrl,
 577 control; CPF; chlorpyrifos; HF, high-fat diet; SC, standard chow diet; *Prkaa1*; AMP-
 578 activated protein kinase catalytic subunit alpha.

579

580 3.4.3. Perinatal CPF exposure is associated with an elevation of liver damage
 581 markers

582

583 The plasma levels of liver enzymes tended to be higher in the offspring of mothers
 584 treated with CPF. This difference was significant (relative increase vs. ctrl-SC: 68%;
 585 p=0.0347) for ALP - one of the most sensitive markers of liver damage (Table 4).

586

587 **Table 4** - Plasma levels of markers of hepatic metabolic activity in 8-week-old male
 588 offspring of dams exposed to CPF and/or an HF diet.

589 The data were expressed as the mean \pm SEM (n=5-20). * statistically significant
 590 difference (p<0.05) vs. ctrl-SCD offspring. ctrl, control; CPF; chlorpyrifos; HF, high-fat
 591 diet; SC, standard chow diet; ALP; Alkaline phosphatase, ALAT; alanine
 592 aminotransferase, ASAT; aspartate aminotransferase, ASAT/ALAT; aspartate
 593 aminotransferase/alanine aminotransferase and LDH; lactate dehydrogenase.

594

	ctrl-SC	CPF-SC	ctrl-HF	CPF-HF
ALP (UI/L)	94.4 ± 21.2	159.1 ± 7.0 *	126.1 ± 7.8	147.6 ± 22.1
ALAT (UI/L)	41.12 ± 9.7	71.79 ± 8.4	77.96 ± 10.7	67.52 ± 4.9
ASAT (UI/L)	116.3 ± 32.1	207.5 ± 35.1	243.0 ± 59.7	251.8 ± 72.0
ASAT/ALAT (UI/L)	2.51 ± 0.3	2.74 ± 0.2	2.84 ± 0.29	3.62 ± 0.9
LDH (UI/L)	539.9 ± 256.1	673.8 ± 103.6	585.8 ± 124.5	1145 ± 343.4

595

596

597 **4. Discussion**

598

599 Perinatal exposure to OPs might be associated with abnormalities in body weight
600 gain and with changes in the expression of certain genes involved in the regulation of
601 glucose and lipid homeostasis in adulthood. Here, we studied the consequences of
602 maternal metabolic disorders and pesticide exposure during critical windows of
603 development on the offspring's metabolism and liver tissue architecture.

604 We exposed the dams to a 1 mg/kg/d dose of CPF which is the oral No Observable
605 Adverse Effect Level in the rat for this pesticide. According to the literature, this dose
606 can depress acetylcholinesterase activity (Cochran et al., 1995). To ensure that all
607 the animals were exposed to the same amount of CPF and to mimic natural
608 exposure in humans (residues on fruits and vegetables), the animals were gavaged.
609 When performed by an experienced handler, gavaging does not generate stress or
610 behavioural or metabolic changes in the dam or the offspring (McDonnell-Dowling et
611 al., 2017). In the HF diet used in the present experiments, 60% of the energy content
612 came from fat; the literature data show that this type of diet induces metabolic
613 alterations in the offspring in adulthood (at 7 months of age) (Lecoutre et al., 2016).

614

615 We found that chronic, repeated exposure of dams to CPF was associated with
616 developmental impairments in the male offspring, as characterized by a lower birth
617 weight and lower plasma levels of growth factors (such as IGF1) and leptin. As leptin
618 is produced mainly in adipose tissue, a low level of this hormone decrease might be
619 linked to low body fat deposition or dysregulated leptin secretion in the male pups.
620 Furthermore, CPF exposure in the dams appeared to induce intrauterine growth
621 restriction, since we observed a significant relative reduction in birthweight. This
622 might be due to CPF's toxic effect on placental nutrition of the foetus, as suggested
623 by studies of rats exposed orally to CPF during the pre- and postnatal periods
624 (Akhtar et al., 2006; Joly Condetto et al., 2015, 2014; Mattsson et al., 2000;
625 Maurissen et al., 2000). Moreover, the CPF exposure in our experiments continued
626 after birth via lactation. It has been reported that CPF concentrates in the mother's
627 milk and that pups are exposed to measurable amounts of CPF during lactation
628 (Mattsson et al., 2000). These impairments appeared to have disappeared by
629 adulthood (PND54), when the offspring were no longer exposed to CPF. In contrast,
630 perinatal exposure to an HFD was associated with greater body weight at weaning

631 and in early adulthood, although the differences vs. control animals were not
632 statistically significant. Our results are in line with those of some other studies
633 (Ribaroff et al., 2017; Tellechea et al., 2017). Even though we did not observe a
634 cumulative effect of perinatal HF diet and CPF exposures, we did find that the HF
635 diet was associated with a significantly greater body weight from birth into young
636 adulthood in pups exposed to CPF - as described previously by other researchers
637 (Cortés-Álvarez et al., 2020). Indeed, the nutritional composition of breast milk is
638 related to maternal obesity and diet during pregnancy and lactation. Ingestion of an
639 HF diet increases the lipid concentration in the mother's milk (Purcell et al., 2011)
640 and modulates the metabolic defects associated with early-life exposure to various
641 OPs. For example, neonatal exposure to parathion and an HF diet was associated
642 with a greater incidence of obesity, diabetes, liver problems and metabolism
643 perturbations in the rat (Adigun et al., 2010; Lassiter and Brimijoin, 2008). In our
644 experiments, maternal CPF exposure was associated with statistically higher plasma
645 levels of insulin and glucose and a clear trend of higher TG. These results were
646 linked to previous studies (Slotkin, 2011; Slotkin et al., 2005a). Unexpectedly, we
647 found that dams in the CPF-HF group were less fertile than the other dams. A recent
648 review reported that an HF diet impairs female reproductive function by altering the
649 hypothalamic-pituitary-ovarian axis (Hohos and Skaznik-Wikiel, 2017), and a late
650 study revealed that CPF also induces reproductive problems (changes in sperm,
651 serum hormone levels, oxidative stress in the testis, and the activity of enzymes
652 related to spermatogenesis) in the rat (Li et al., 2019), ~~we did not observe such~~
653 ~~consequences in our model~~. Since all the animals in the present study were raised in
654 the same environment, the observed difference can only be explained by the
655 cumulative effect of the two harmful factors studied here. To clarify this point, the
656 cumulative impacts of CPF and an HFD on reproductive functions (with a focus on
657 putative follicular atresia in the dams' ovaries) shall be investigated further.

658

659 The low weight gain described above was correlated with alterations in lipid and
660 glucose metabolism. Perinatal exposure to CPF or an HF diet was associated with
661 lower expression of key genes in lipogenesis, such as *Acaca* (coding for an enzyme
662 that produces malonyl-CoA for fatty acid synthesis) and *Fasn* (coding for an enzyme
663 that catalyses the last step in fatty acid biosynthesis). In contrast, expression of
664 *Srebf1/Srebp1c* (coding for the main transcription factor for *Acaca* and *Fasn*) was

665 only affected by HF diet exposure. Perinatal exposure to CPF or an HF diet also led
666 to a relative decrease in the expression of lipid accumulation markers such as *Cd36*,
667 *Dgat 1*, and *Dgat2*; the latter two enzymes catalyse the final step in TG synthesis.
668 Although this decrease might conceivably lead to a lower TG content in the liver, we
669 actually observed a slight increase. We hypothesize that fewer fatty acids were
670 synthesized from acetyl CoA and so fewer TGs were stored in the liver. This
671 difference was associated with greater expression of *Lpl* following perinatal CPF
672 exposure. ~~*Lpl* hydrolyses TGs into fatty acids and glycerol and is also a ligand~~
673 ~~facilitating the cellular uptake of VLDL and LDL. This could be responsible for an~~
674 ~~increase in lipid transport. As a consequence, we could expect an increase of *Cd36*~~
675 ~~mRNA expression related to lipid-rich diets and hepatic steatosis (Miquilena-Colina et~~
676 ~~al., 2011).~~ But we did not notice this increase in progeny from mother exposed to
677 CPF or HFD, indicated a normal state of hepatocytes. Moreover, perinatal exposures
678 to CPF alone or an HF diet alone were both associated with greater mRNA
679 expression of *Pparg* (a nuclear receptor and transcription factor involved in lipid
680 storage) in the male pups. Likewise, perinatal HF diet exposure was also associated
681 with lower mRNA expression of *Hmgcr* - the main gene related to lipid (cholesterol)
682 synthesis - in these animals. The lower level of hepatic lipid storage in the offspring
683 exposed to CPF and HFD was probably due an overall reduction in lipid metabolism,
684 as suggested by the lower gene expression of key factors. However, it remains to be
685 seen whether protein expression and function are modified. The mechanism(s)
686 underlying differences in gene expression will also have to be characterized. A
687 change in protein levels would indicate dysregulation of the main hepatic lipogenic
688 pathways and thus an impairment in hepatic metabolism. In line with the literature
689 data, concomitant perinatal co-exposure of the dams to both CPF and HFD tended to
690 have the same consequences as each factor separately; although synergistic effects
691 were not observed (Adigun et al., 2010).

692
693 Maternal CPF exposure was associated with greater mRNA expression of *Ppara* in
694 the pups' liver, whereas maternal HF diet exposure was associated with lower
695 expression of the gene in its offspring. *Ppara* is involved in the inflammatory
696 response and in fatty acid oxidation. *Ppara* targeted genes control the release of fatty
697 acids from TG-rich lipoproteins, chylomicrons, and VLDL. In our study, the lower
698 plasma TG level reflected lower hepatic synthesis and greater catabolism in the

699 plasma; this may have been related to regulation of the *Lpl* gene. This hypothesis
700 was confirmed by the significantly greater mRNA expression of *Lpl* and *Ppara* genes
701 and the elevation (albeit non-significant) in the plasma TG level in the offspring of
702 CPF-exposed dams. Perinatal CPF and HF diet exposure was also associated with
703 differences in glucose metabolism. We noted lower expression of *Slc2a2/Glut2* (a
704 glucose transporter) and *G6pc* (a major regulatory enzyme in gluconeogenesis and
705 which catalyses the hydrolysis of glucose-6-phosphate to D-glucose). A lower level of
706 gluconeogenesis might be related to the elevation (albeit non-significant) in
707 glycaemia after perinatal exposure of dams to CPF or an HF diet. Moreover, insulin
708 inhibits the transcription of *Pck1/Pepck* gene, which is involved in gluconeogenesis
709 and the transformation of oxaloacetate into phosphoenolpyruvate. Perinatal HFD
710 exposure was associated with lower *Pck1/Pepck* gene expression in the offspring.
711 We hypothesize that lower glucose uptake by liver cells resulted in an elevation of
712 glycaemia and a relative decrease in lipid synthesis in general and TG synthesis in
713 particular.

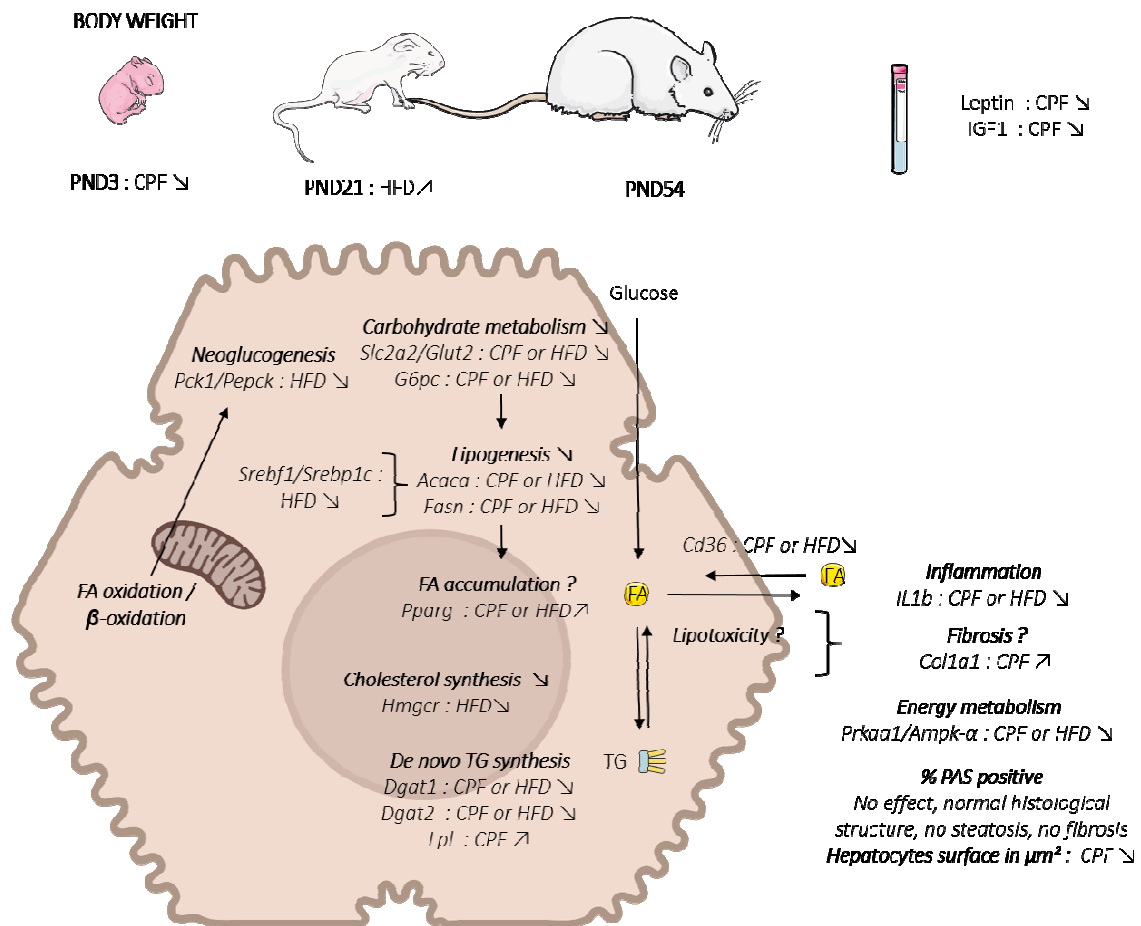
714

715 We also looked at potential tissue changes and inflammation in the liver. The plasma
716 levels of liver enzymes went higher after perinatal CPF exposure (relative to
717 controls), with a relative reduction in hepatocyte size (in agreement with the literature
718 (Mansour and Mossa, 2010)); these differences were suggestive of damage to the
719 hepatocyte membrane. The relative increase associated with CPF exposure was
720 statistically significant for ALP - the most sensitive marker of liver damage. No
721 histological alterations in young adult rats were noted in any of the experimental
722 groups. Maternal exposure to CPF was also associated with greater expression of
723 the *Col1a1* gene (coding for one of the two main components of collagen fibres); this
724 might predict the subsequent development of fibrosis. In contrast, maternal exposure
725 to an HF diet was not associated with any differences in markers of liver damage in
726 the offspring, except a slight but nonsignificant elevation of plasma levels of liver
727 enzymes. Nevertheless, concomitant maternal exposure to both an HF diet and CPF
728 was associated with lower mRNA expression of *Prkaa1/Ampka*, a sensor of the cell's
729 energy homeostasis. This enzyme switches on fatty acid oxidation and glycolysis and
730 switches off lipogenesis via short-term effects on regulatory protein phosphorylation
731 and long-term effects on gene expression (Foretz et al., 2006). We expected to
732 observe greater expression of this gene in the offspring of dams exposed to both an

733 HF diet and CPF; this would have explained the lower level of lipogenesis. The
734 observed impacts might only be predictive of more severe impairments later in life;
735 the age of 8 weeks was a probably too early to observe such metabolic effects. Even
736 though the exact fat content of an HF diet is not correlated with any outcomes in
737 offspring, other factors (such as the species, the effects of the interventional diet on
738 the mother, the intervention time point, and the micronutrient and macronutrient
739 content of the control diet) might account for the differences in outcomes between
740 studies in which HF diets were used to induce obesity (Ribaroff et al., 2017).

741

742 The primary objective of the present study was to assess the potential impact of a
743 harmful maternal environment (an obesogenic diet and/or exposure to the pesticide
744 CPF) in the offspring. Even in the absence of direct contact between the offspring
745 and the harmful factors (except through lactation), we found that exposure had an
746 impact on metabolic activity in the liver. Indirect exposure to CPF was associated
747 with alterations in the mRNA expression of several liver enzymes, which in turn might
748 have reduced neoglucogenesis and *de novo* lipogenesis. Exposure to an HF diet
749 alone during the perinatal period induced the same type of effect as CPF alone -
750 showing that long-term metabolic disorders can occur in the absence of inflammatory
751 signs (Figure 11).



752

753 **Figure 11** – Hypothetical changes in the liver during the development of the male
 754 offspring of dams exposed to CPF alone and/or combined with an HFD. CPF;
 755 chlorpyrifos; HFD, high-fat diet; *Acaca*, acetyl-CoA carboxylase 1; *Cd36*; fatty acid
 756 translocase; *Col1a1*; collagen alpha-1; *Dgat1*; Diacylglycerol O-acyltransferase 1;
 757 *Dgat2*; Diacylglycerol O-acyltransferase 2; FA, fatty acids; *Fasn*, fatty acid synthase;
 758 *G6pc*, glucose-6-phosphatase; *Hmgcr*; 3-hydroxy-3-methyl-glutaryl-coenzyme A
 759 reductase; *IL1b*; interleukin 1 beta; *Lpl*; lipoprotein lipase; *Pck1*,
 760 phosphoenolpyruvate carboxykinase; *Ppara*, peroxisome proliferator activated
 761 receptor alpha; *Pparg*, peroxisome proliferator activated receptor gamma; *Prkaa1*;
 762 AMP-activated protein kinase catalytic subunit alpha; *Slc2a2*, solute carrier family 2,
 763 facilitated glucose transporter member 2; *Srebf1*, sterol regulatory element binding
 764 factor 1 variant 2.

765

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775

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