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### ► To cite this version:

Marion Guibourdenche, Hiba El Khayat El Sabbouri, Fidéline Bonnet, Narimane Djekkoun, Hafida Khorsi-Cauet, et al.. Perinatal exposure to chlorpyrifos and/or a high-fat diet is associated with liver damage in male rat offspring. *Cells & Development*, 2021, 166, pp.203678. <10.1016/j.cdev.2021.203678>. <hal-03214318>

**HAL Id: hal-03214318**

**<https://hal.science/hal-03214318v1>**

Submitted on 9 May 2023

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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 *Mlx1pl/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).

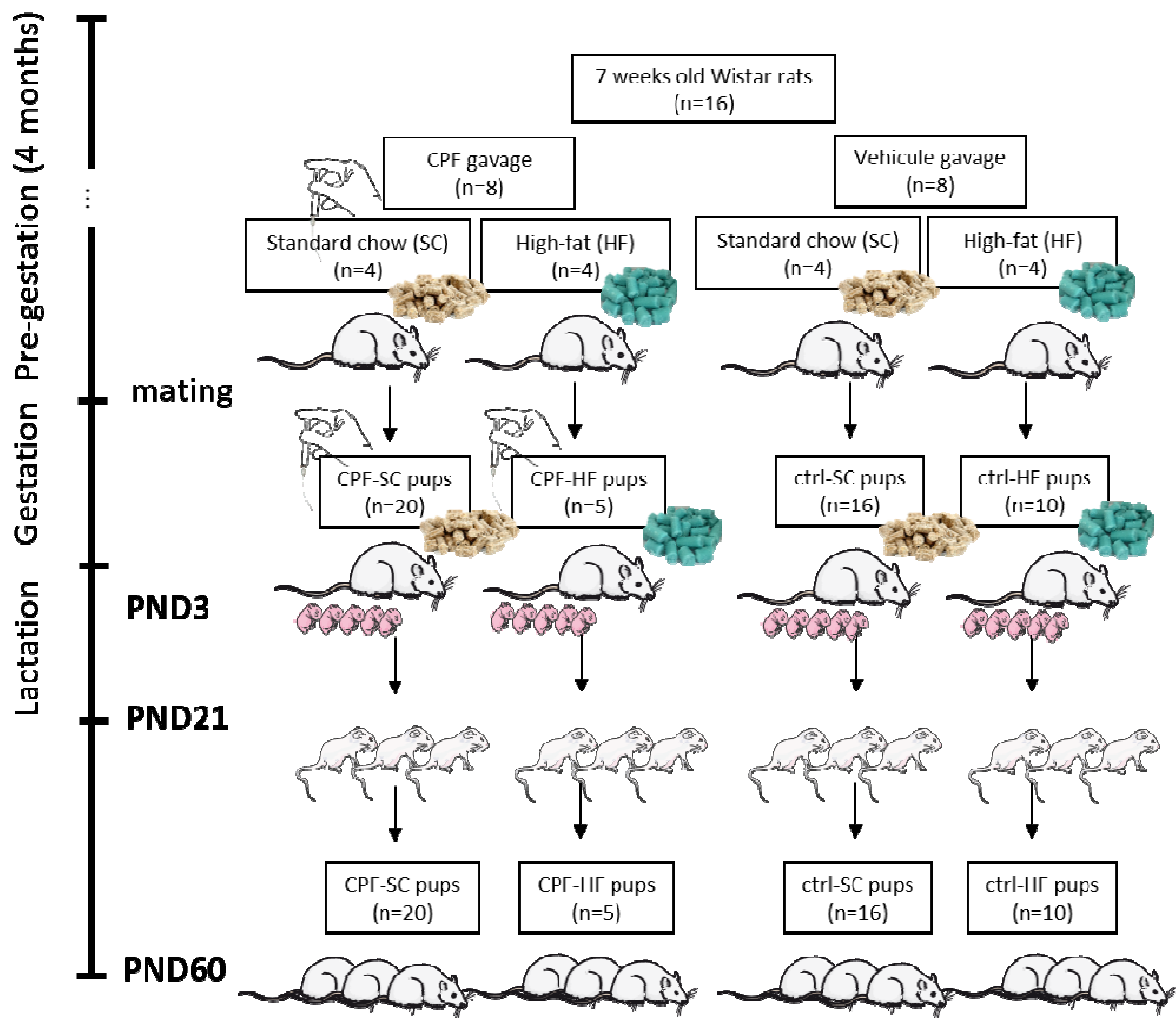
170 The animals' welfare was monitored daily. All experimental protocols had been  
171 audited by the local animal care and use committee (C2EA#96, Amiens, France) and  
172 approved by the French Ministry of Research (reference: APAFIS # 8207-  
173 2016121322563594 v2). All animal experiments complied 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:  $225 \pm 17$  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

| <b>Crude nutrients (%)</b> | <b>HF diet<sup>a</sup></b>                         | <b>SC diet</b>                   | <b>Standard breeding chow diet<sup>c</sup></b> |
|----------------------------|--|----------------------------------|--|
| <b>Product reference</b>   | D12492   | 3436                             | 3336   |
| <b>Supplier</b>            | Ssniff<br>Spezialdiäten<br>GmbH, Soest,<br>Germany | Serlab,<br>Montataire,<br>France | Serlab,<br>Montataire,<br>France               |

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

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

|                               |         |         |         |
|-------------------------------|---------|---------|---------|
| <b>Folic acid</b>             | 16 mg   | 2 mg    | 2.4 mg  |
| <b>Biotin</b>                 | 300 µg  | 220 µg  | 220 µg  |
| <b>Choline</b>                | 920 mg  | 2000 mg | 2000 mg |
| <b>Vitamin C</b>              | Unknown | 40 mg   | 40 mg   |
| <b>Trace elements (mg/kg)</b> |         |         |         |
| <b>Iron</b>                   | 168     | 250     | 200     |
| <b>Zinc</b>                   | 65      | 60      | 68      |
| <b>Copper</b>                 | 13      | 14      | 15      |
| <b>Iodine</b>                 | 1.2     | 1       | 1       |
| <b>Manganese</b>              | 95      | 60      | 40      |
| <b>Selenium</b>               | 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:  $316 \pm 33$  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  $10 \pm 5$  pups, with  $4 \pm 3$   
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<sup>later</sup> 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

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

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

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

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

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](http://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.

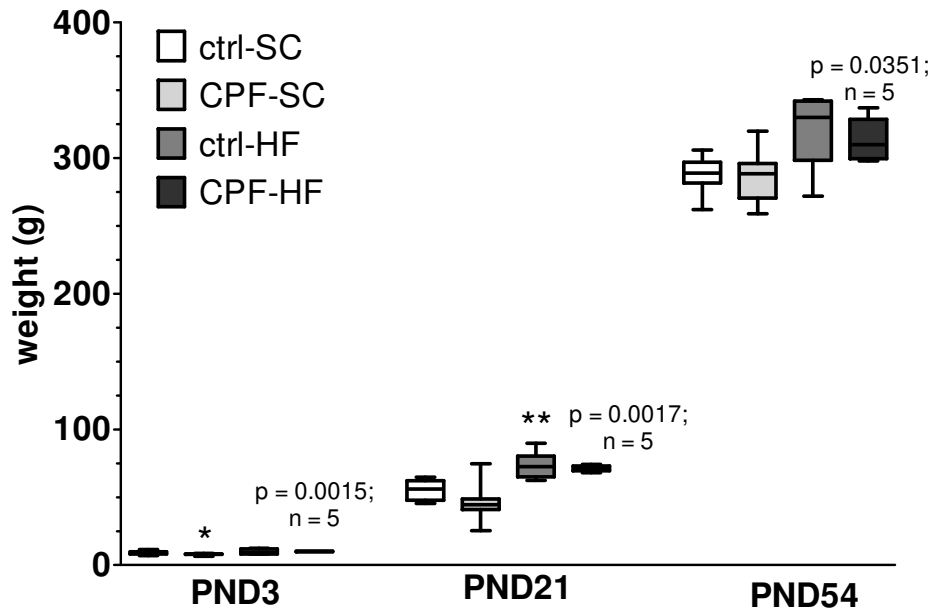
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### 3. Results

#### 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

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

|                                 |             |                |             |             |
|---------------------------------|-------------|----------------|-------------|-------------|
| <b>Fasting IGF1<br/>(ng/mL)</b> | 6.47 ± 0.33 | 3.74 ± 0.57 ** | 6.00 ± 0.50 | 4.61 ± 1.73 |
|---------------------------------|-------------|----------------|-------------|-------------|

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

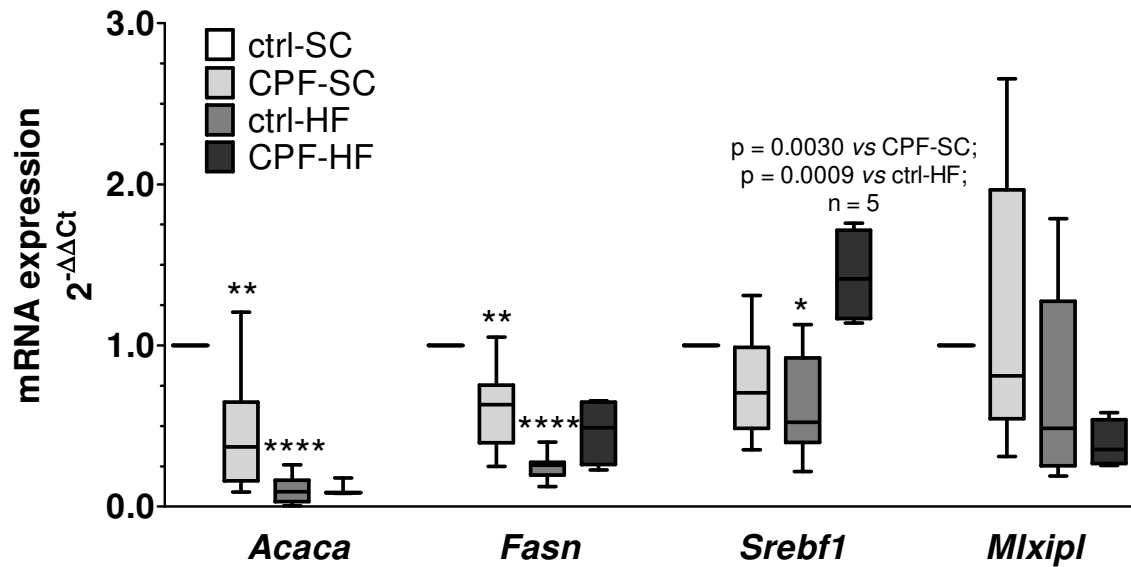
406

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



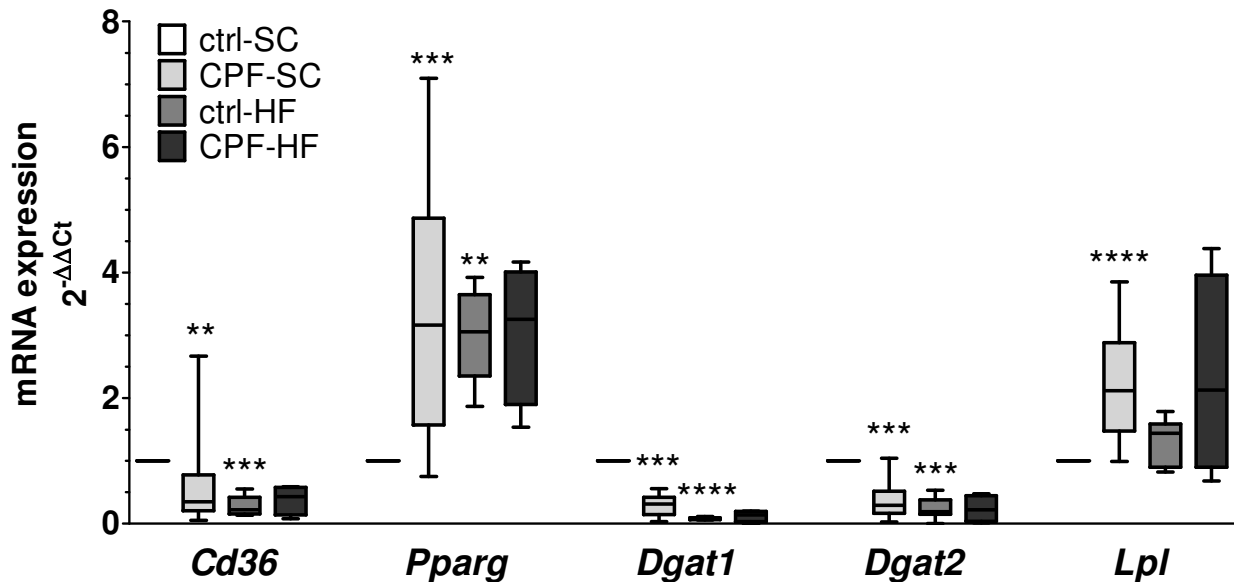
424  
 425 **Figure 3** – mRNA expression (RT-PCR) of lipogenesis markers in 8-week-old male  
 426 offspring of dams exposed to CPF and/or an HF diet.

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).



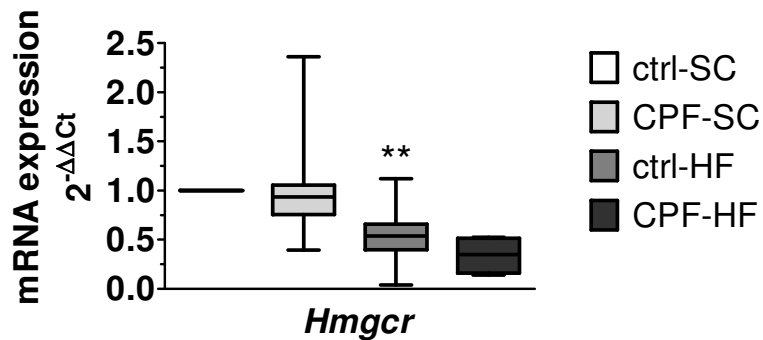
450

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<sup>-ΔΔCt</sup> 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.

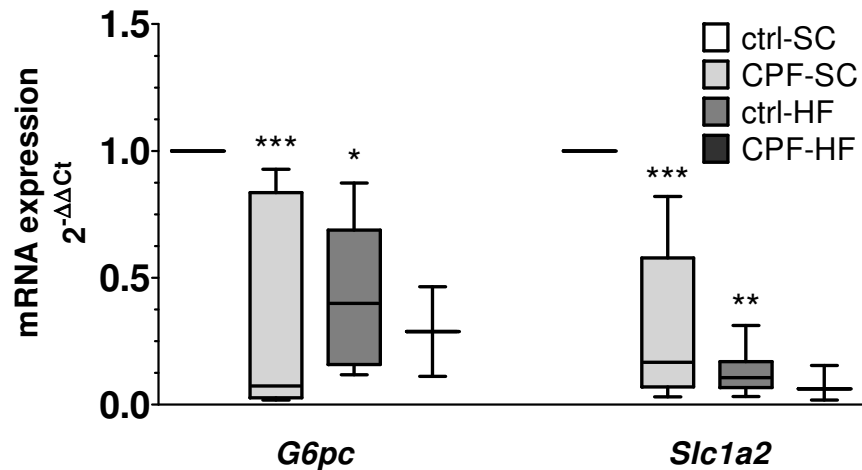
469 The mRNA gene expression levels were quantified as the fold induction, relative to  
 470 the control group (set to 1). Data were expressed as the mean  $\pm$  SEM (n=5-20), using  
 471 the  $2^{-\Delta\Delta C_t}$  calculation method with the *Gapdh* housekeeping gene. \*\* statistically  
 472 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).



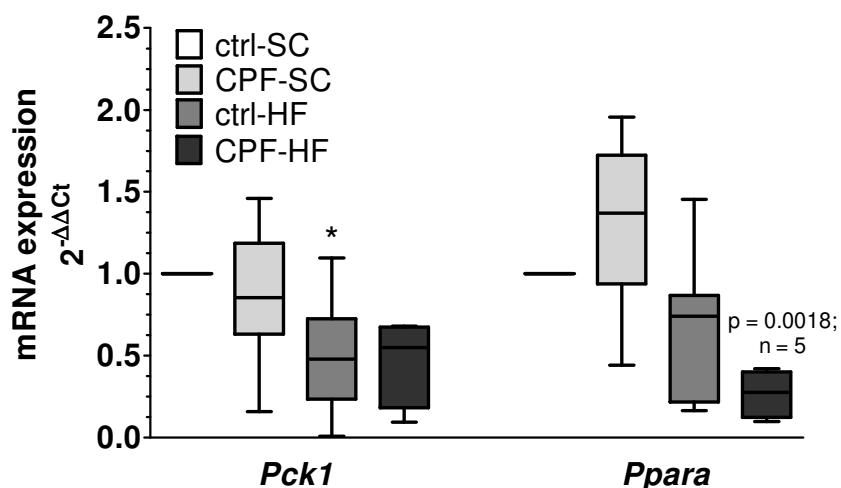
486

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

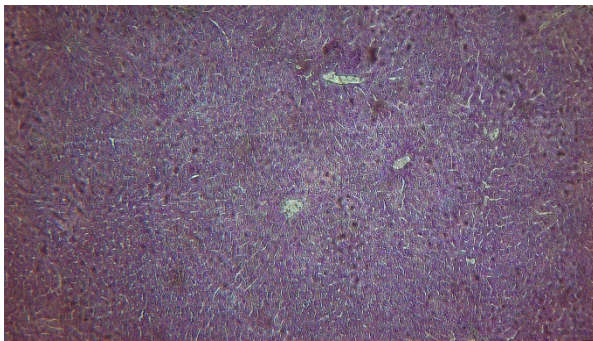
527 the CPF-SCD offspring than in the ctrl-SCD offspring. No collagen fibres could be  
528 seen between the hepatocytes – suggesting that the amount of collagen was below  
529 the staining methods' limits of detection. Therefore, we did not observe any  
530 characteristic features of hepatic fibrosis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

531

**A**

**PAS staining**

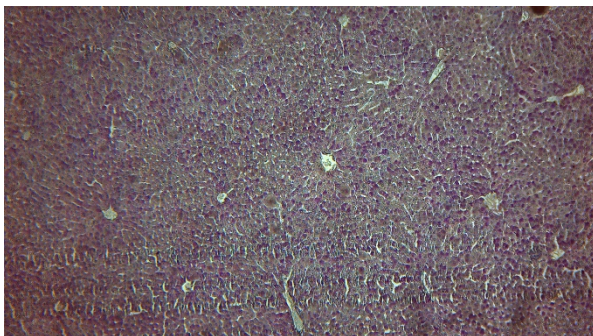
**ctrl-SC**



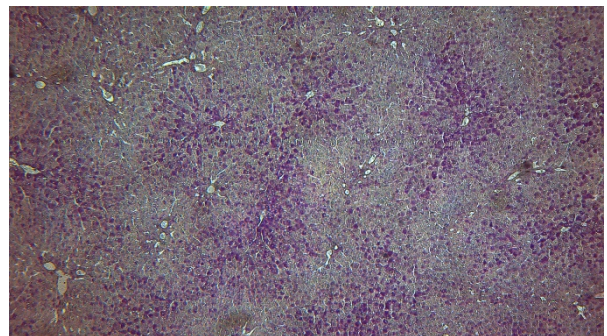
**CPF-SC**



**ctrl-HF**



**CPF-HF**



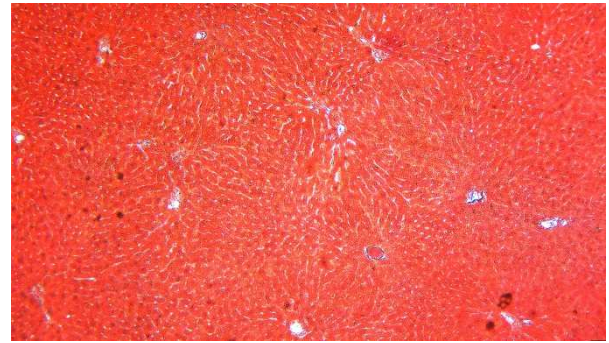
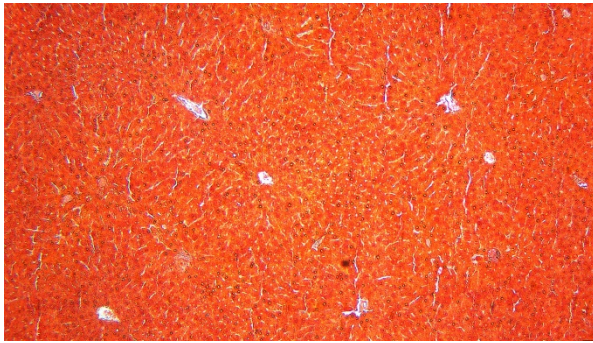
532

**B**

**Masson's trichrome staining**

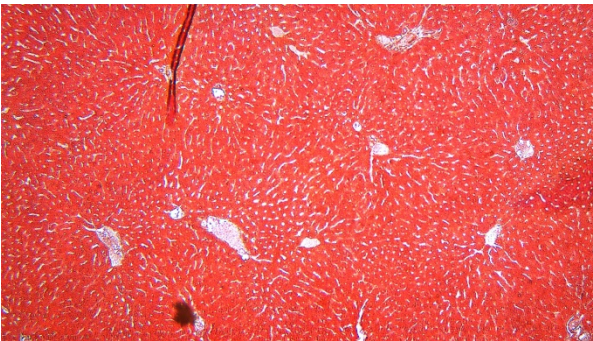
**ctrl-SC**

**CPF-SC**



**ctrl-HF**

**CPF-HF**

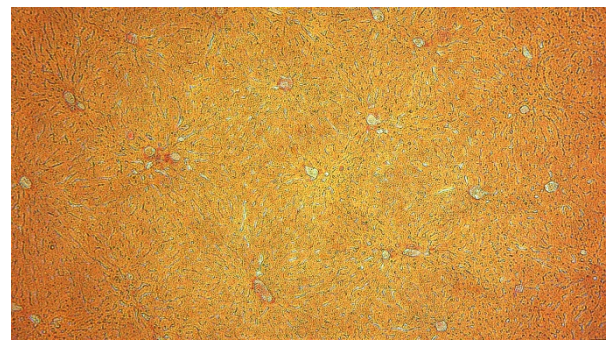
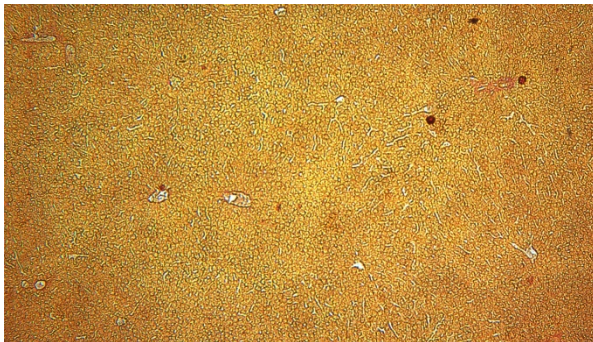


**C**

**Sirius Red staining**

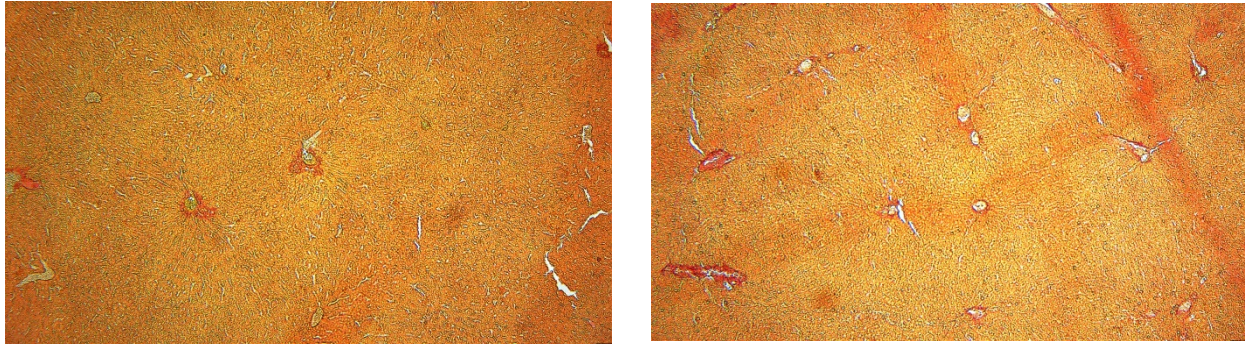
**ctrl-SC**

**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.

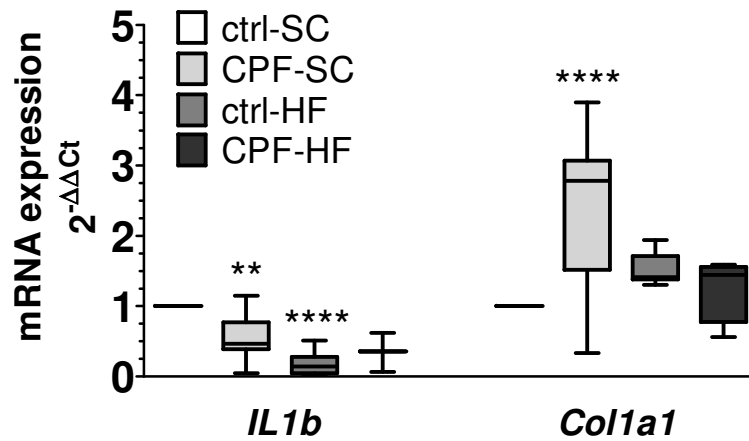
542

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

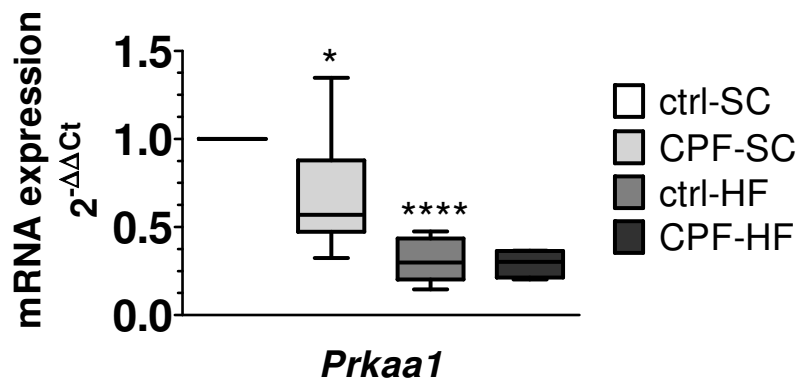
554 **Figure 9** - mRNA expression (RT-PCR) of markers of inflammation and fibrosis in 8-  
 555 week-old male offspring of dams exposed to CPF and/or an HF diet.

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

|                             | <b>ctrl-SC</b> | <b>CPF-SC</b> | <b>ctrl-HF</b> | <b>CPF-HF</b> |
|-----------------------------|----------------|---------------|----------------|---------------|
| <b>ALP (UI/L)</b>           | 94.4 ± 21.2    | 159.1 ± 7.0 * | 126.1 ± 7.8    | 147.6 ± 22.1  |
| <b>ALAT (UI/L)</b>          | 41.12 ± 9.7    | 71.79 ± 8.4   | 77.96 ± 10.7   | 67.52 ± 4.9   |
| <b>ASAT (UI/L)</b>          | 116.3 ± 32.1   | 207.5 ± 35.1  | 243.0 ± 59.7   | 251.8 ± 72.0  |
| <b>ASAT/ALAT<br/>(UI/L)</b> | 2.51 ± 0.3     | 2.74 ± 0.2    | 2.84 ± 0.29    | 3.62 ± 0.9    |
| <b>LDH (UI/L)</b>           | 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.

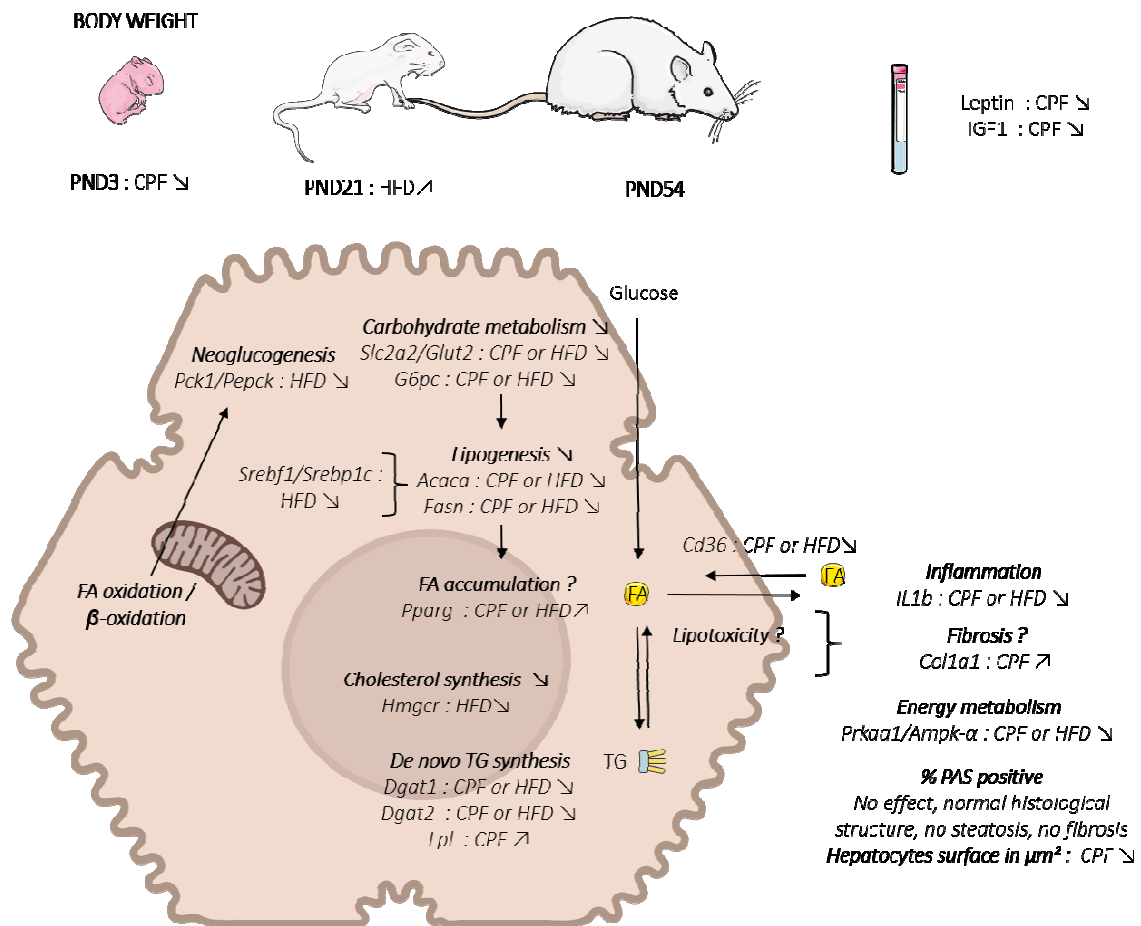
714

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

766 **Acknowledgement:**

767 The authors thank J. Le Ber and her staff in the animal facility for their help  
768 monitoring animals and Dr. D. Fraser (Biotech Communication SARL,  
769 Ploudalmézeau, France), for his extensive English editing.

770

771 **Funding:**

772 This work was funded by the University of Picardie Jules Verne, the Hauts-de-France  
773 Regional Council, and the Hospital-University Federative Project “1000 Days for  
774 Health” (PROGHOMEO grant – 2017).

775

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