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1 **Aerobic metabolism and cardiac activity in the descendants of zebrafish exposed to**
2 **pyrolytic polycyclic aromatic hydrocarbons**

3
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14
15 **Abstract**

16 The increase of anthropogenic activities on coastal areas induces discharges of Polycyclic Aromatic
17 Hydrocarbons (PAHs) in aquatic ecosystem. PAHs effects depend on their concentration and the way of
18 contamination, but also on the different developmental stages of the organism. Zebrafish were exposed to
19 relevant concentration of pyrolytic PAHs from the first meal (i.e. 5 day post fertilization, dpf) to mature adults.
20 Parental effect of this type of exposure was evaluated through the assessment of aerobic metabolic scope, cardiac
21 frequency and cardiac mRNA expression on larval and/or embryo progeny of contaminated fish. Our results
22 suggest that cardiac frequency increased in larval descendants of fish exposed to the environmental
23 concentration of pyrolytic PAHs (i.e. 5ng.g⁻¹ of food), while a lack of effect on aerobic metabolism in 5 dpf
24 larvae was highlighted. A surexpression of mRNA related to the cardiac calcium transporting ATPase *atp2a2a*, a
25 protein essential for contraction, is in accordance with this increasing cardiac frequency. Even if cardiac
26 development genes *cmlc1* and *tmt2a* were not affected at early lifestages tested, complementary work on cardiac
27 structure could be interesting to better understand PAHs action.

28
29 *Keywords:* parental transfer, pyrolytic hydrocarbons, sub-lethal concentration, metabolic rate, heart

44 **Introduction**

45

46 Polycyclic aromatic hydrocarbons (PAHs) constitute an important family of persistent organic pollutants (POPs)
47 that may be issued from natural and anthropogenic sources. Pyrolytic PAHs are released through partial
48 combustion of organic matter at high temperature (Hylland et al. 2005) and enter in aquatic ecosystem through
49 atmospheric deposition. These types of PAH are mainly characterized by high molecular weight PAHs (four- to
50 six-rings) and a small proportion of alkylated compounds (e.g. methylated compounds). On the contrary, natural
51 discharges of petroleum or oil accidents introduce petrogenic PAHs, which are dominated by low-molecular
52 weight compounds (two- to four- rings) and alkylated compounds. In general, PAHs are characterized by their
53 resistance and ubiquity (Dabestani and Ivanov 1999). Found as complex mixtures in environment, they are
54 related to multiple mechanisms of toxicity driven by their chemical properties, their concentration, and their
55 origins. All compounds in PAHs mixture could have synergic or antagonist effects depending on their chemical
56 properties and biotransformation (Barron 1999; Van der Oost et al. 2003; Wassenber and Di Giulio 2004; Billiard
57 et al. 2008). In addition to the risk for human health, PAHs are well described in literature especially for their
58 carcinogenic, genotoxic and mutagenic effects on fish (Hawkins et al. 2003; Myers et al., 1991; Tuvikene, 1995).
59 PAHs are also known to impact growth, reproduction and survival of aquatic organisms (Meador et al. 2006;
60 Kim et al. 2008; Horng et al. 2010). The lipophilic property of PAHs induces their adsorption by suspended
61 particles in aquatic ecosystem, (Fowler 1993; Latimer and Zheng, 2003). Consequently, aquatic organisms may
62 be affected by various pathways such as aqueous exposure, but also sediment contact for instance.

63 Some studies show that PAHs have different effects on organisms. They could for instance affect the
64 reproductive function (Collier *et al.*, 1992; Seruto *et al.*, 2005) through reduction or inhibition of gonads
65 development, decrease of the eggs quality and number, as well as reduction of egg and larval viability (Hall and
66 Oris 1991, Von Westernhagen et al. 1981; Fletcher et al. 1982). Moreover, PAHs or their derivatives could be
67 accumulated in parent's gonads and transferred into the eggs. This precocious exposure to PAHs can be
68 deleterious for descendants and reduce the offspring survivorship. Hall and Oris (1991) observed that a maternal
69 exposure to anthracene induced teratogenic effects (e.g. oedema, eyes and yolk deformities) on the progeny of
70 fathead minnows (*Pimephales promelas*). PAHs-indirect effects can be also considered. Recent studies have
71 indeed shown that maternal stress influences offspring characteristic through the production of stress hormones,
72 such as cortisol. Level of cortisol is commonly used as an indicator of the degree of stress experienced by fish
73 (Barton, 2002; Barton and Iwama, 1991; Wendelaar Bonga, 1997 ; Aldegunde *et al.*, 1999; Kennedy and Farrell,
74 2005; Tintos *et al.*, 2008; Marentette *et al.*, 2013). Thereby, pollutants could disrupt physiological processes
75 regulated by cortisol, such as metabolism or reproduction (Gesto *et al.*, 2008; Tintos *et al.*, 2007, 2008). In
76 addition, maternal cortisol produced under stressor could be accumulated in eggs and affect the gametes quality
77 (Campbell *et al.*, 1992, 1994), the size or the quality of the progeny of fish (McCormick 1998, 1999, 2006).

78

79 In this context, the main objective of this study was to investigate the effects of pyrolytic contamination through
80 parental transfer. The interest of this study come from the lack of investigation and understanding regarding the
81 effect of parental PAH-exposure on the descendants. In order to do so, aerobic metabolism and cardiac frequency
82 were measured in 5 days post fertilizations (dpf) larvae born to genitors exposed to pyrolytic-PAHs through
83 trophic pathway. Furthermore, to have a better view of the cardiac effects in the descendants of zebrafish
84 exposed to PAHs, genes expression in relation to heart development and activity (Nesan and Vijayan 2012) were

85 measured in embryo (48 hours post-fecundation, hpf) and larvae (6dpf).

86

87 **Material & Methods**

88

89 *Protocol of food contamination*

90

91 Pyrolytic PAHs fraction (*PY*) has been extracted from sediment in the Seine Bay (France). According to the
92 protocol described by Budzinski et al. (1997) and Cachot et al. (2007), the 16 most toxicants PAHs listed by the
93 US Environmental Protection Agency (US-EPA) have been extracted and quantified. Age-adapted food (INICIO
94 Plus, Biomar, France) was then spiked with *PY* fraction using protocol adapted from Vicquelin et al. (2011) and
95 described in Vignet et al. (in press). The concentration $5\mu\text{g}\cdot\text{g}^{-1}$ of food was considered as an environmental
96 relevant value, since it is representative of what is measured in mollusks in the Seine bay (Cachot et al., 2006).
97 Based on this concentration of reference called *X*, three concentrations were targeted in the experimental food :
98 *0.3X*, *1X* and *3X*. A fourth treatment was tested for which the experimental food contained only dichloromethane,
99 the solvent used to spike the food. This treatment was considered as the *control*. PAHs compositions and
100 concentrations in diet used for exposure are described in detail in Vignet et al. (in press). Briefly, the total
101 concentration of PAHs ($\text{ng}\cdot\text{g}^{-1}$ dry weight food) for treatments *control*, *0.3X*, *1X*, *3X* was respectively 55 ± 12 ,
102 $1,763 \pm 0,468$, $5,816 \pm 1,433$, $18,151 \pm 4.983$.

103

104 *Zebrafish exposure*

105

106 The tested progeny came from genitors directly exposed to *PY*- PAHs by trophic pathway. These genitors were
107 themselves obtained from uncontaminated couples (wild-type Tuebingen strain, *TU*) as described in Vignet et al.
108 (in press). Genitors were fed from the first meal (5 dpf) to the adult stage by one of the 4 types of food (i.e.
109 *control*, *0.3X*, *1X* or *3X*). These groups of contaminated individuals represented the first generation called F_0 .
110 When they were mature (i.e. at 4 months old), some of these fish were used to form couples to obtain a new
111 generation called F_1 . Until 5dpf, embryos and larvae F_1 were maintained in Petri dishes (6-7 ml) into ionic
112 enriched solution of E3 at a constant temperature of 28°C and a photoperiod of 14 h light/10 h dark. Because of
113 enough yolk sac reserves that fulfill dietary needs and the mouth opening, that occurs at the age of 5 dpf, larvae
114 never received food before the test. The tests were made on these 5 dpf, larvae.

115

116 *Metabolites quantification*

117

118 PAHs metabolites were extracted from three pools of 5dpf larvae F_1 for each treatment. Larvae were
119 homogenized in 3-4 mL of acetate buffer solution ($\text{pH} = 5.0$). After the addition of internal calibration
120 compounds and beta-glucuronidase enzymes, these samples were placed at 37°C and submitted to an enzymatic
121 deconjugation during 16 hours. Metabolites were extracted by Solid Phase Extraction (SPE) procedure using
122 octadecyl cartridge (C_{18} , Bakerbond, 500 mg, 3cc, elution with 100 % of methanol) as described by Mazéas
123 (2005). The organic extract obtained was then concentrated under gas flow and purified by amino column (NH_2 ,
124 Supelco, 500 mg, 3cc, elution with a mix of 80/20, v/v, dichloromethane/methanol). This final extract obtained
125 was quantified by liquid chromatography associated coupled to tandem mass spectrometry (LC/MS/MS) as
126 described by le Dû-Lacoste (2013). PAHs metabolites in larvae are described in the Table 1.

Table 1 Metabolites concentrations in larvae F₁ from genitors contaminated with pyrolytic PAHs. Mean \pm SD in ng.g⁻¹ of larvae, n.d. not detected.

Metabolites	Control	0.3X	1X	3X
Σ Hydroxynaphthalene (1+2 OHNaph)	nd	nd	nd	nd
2 Hydroxybiphenyle (2OHBi)	<blank	<blank	12	16.1
Σ Hydroxyphenanthrene (1+2+3+4+9 OHPhe)	<blank	0.3	0,8	1
3 Hydroxyfluoranthene (3OHFluo)	nd	nd	nd	nd
1Hydroxypyrene (1OHPyr)	2	7	15.3	84.3
1 hydroxychrysene (1OHChrys)	nd	nd	1.5	nd
Σ Hydroxybenzo(a)pyrene (3+9 OHBaP)	nd	nd	9.1	34.6
Sum Hydroxy-PAHs	2	7.3	39	136

127

128

129 *Respirometry*

130

131 Experimental set-up

132 The set-up consisted of 8 independent glass micro respirometer chambers (diameter $d= 1.12$ cm, volume $V=$
 133 $0.98.10^{-3}$ l; Loligo systems, Denmark). Respirometers were submerged into buffer tanks (depth x length x height:
 134 10x20x31 cm) and filled with oxygenated E3 solution at a constant temperature of 28°C. Peristaltic pumps
 135 controlled the water supply from the buffer tank to each respirometer. Each pump was controlled by a timer,
 136 allowing intermittent flow respirometry (Steffensen 1989), where phases of oxygen renewal alternated with
 137 phases of oxygen consumption measurements with a period of 15:15 min. Each respirometer was equipped with
 138 a fiber optic sensor (PreSens) connected to a multichannel oxygen measuring system (OXY 4 mini, PreSens) to
 139 record dissolved oxygen levels. Optic fibers were calibrated at 0% and 100% of air saturation at a temperature of
 140 28°C. A factor of conversion based on oxygen solubility into water was used to convert oxygen data from
 141 percentage saturation to mgO₂.l⁻¹ (i.e. 100% was equivalent to 7.94 mgO₂.l⁻¹ for a 28°C temperature and a 0
 142 salinity). Oxygen saturation was recorded every five seconds with the program Oxyview (PreSens).

143

144 Experimental protocol

145 Each experimental trial consisted in testing simultaneously 4 groups of 10 larvae F₁ whose genitors were
 146 exposed to one of the four treatments (0.3X, 1X, 3X and control). A total of 36 groups was tested ($n_{control}=9$
 147 groups, $n_{0.3X}=8$ groups, $n_{1X}=12$ groups, $n_{3X}=7$ groups). Each experimental trial involved two steps. First, to assess
 148 active metabolic rate (AMR), metabolism of larvae F₁ was increased through chasing (Schurmann and Steffensen
 149 1997; Lefrançois and Claireaux 2003; Jourdan-Pineau et al. 2010; Cannas et al. 2013, Clark et al. 2012). The
 150 tested groups of 10 larvae were chased with a stick into petri dishes. When exhausted (i.e. when fish did not
 151 respond to stimulation), each group was transferred into one of the 4 respirometers to assess AMR through a first
 152 oxygen consumption over the next 15 min. Second, to estimate standard metabolic rate (SMR), larvae were left
 153 undisturbed in the respirometer for 48h, during which oxygen consumption was regularly and automatically
 154 measured. After this period of measurements, larvae were removed from the respirometers and slightly

155 anesthetized with benzocaine at a concentration of 50mg.l⁻¹. The body mass of each individual was determined
156 using a microbalance. The tested groups showed homogenous weights; the average 'group weight' being $M=$
157 0.20 ± 0.02 mg (mean \pm S.E). Before and after each trial, a blank measurement was performed to quantify
158 microbial oxygen consumption in the respirometer. The average of these 2 values was subtracted from the
159 measured oxygen consumption. Each group of larvae was tested once.

160

161 Calculations

162 Oxygen consumption MO_2 is expressed in $mgO_2 \cdot g^{-1} \cdot h^{-1}$ and calculated according to the following formula:

163

$$164 \quad MO_{2meas} = \Delta[O_2] \cdot V \cdot \Delta t^{-1} \cdot M_{meas}^{-1} \quad \text{equation (1)}$$

165

166 where $\Delta[O_2]$ (in $mgO_2 \cdot l^{-1}$) is the variation in oxygen concentration during the measurement period Δt (in h) and V
167 (in l) is the volume of the respirometer minus the volume of the group of 10 larvae, M_{meas} (in g) is the mass of
168 the corresponding tested group of 10 larvae .

169

170 An allometric relationship exists between oxygen consumption and body mass, which permitted to correct
171 MO_{2meas} using the following formula:

172

$$173 \quad MO_{2cor} = MO_{2meas} \cdot (M_{meas} \cdot M_{cor}^{-1})^{1-b} \quad \text{equation (2)}$$

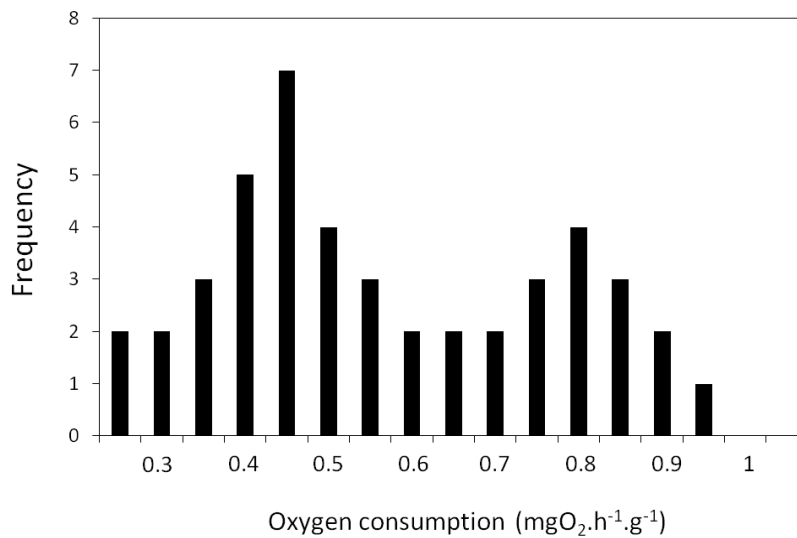
174

175 where MO_{2cor} (in $mgO_2 \cdot g^{-1} \cdot h^{-1}$) is the oxygen consumption related to a standard fish of 1g (M_{cor}), MO_{2meas} (in
176 $mgO_2 \cdot g^{-1} \cdot h^{-1}$) is the oxygen consumption estimated for experimented fish whose mass was $M_{(meas)}$ (in g) and b is
177 the allometric scaling exponent describing the relationship between oxygen consumption and body mass of fish.
178 We used $b_{AMR}=0.926$ for the correction of active metabolic rate and $b_{SMR}=0.965$ for the correction of standard
179 metabolic rate (Lucas et al., 2014).

180

181 AMR was assessed as the maximal MO_2 obtained after chasing the larvae. SMR was estimated according to the
182 method described by Steffensen et al. (1994). Briefly, the frequency distribution of MO_2 values recorded during
183 the last 24 hours of the test was plotted. This generally produces a bimodal frequency distribution due to the
184 routine activity of the fish. The higher mode (*i.e.* the first peak, Fig. 1) is considered to reflect the SMR and the
185 lower mode (*i.e.* the second peak, Fig. 1) corresponds to the routine metabolic rate (RMR), *i.e.* the energy
186 required by the fish for spontaneous activity. Aerobic metabolic scope AMS was calculated as the difference
187 between AMR and SMR . AMR , SMR and AMS were assessed for each group.

188



189
 190 Fig. 1 An example of frequency distribution of oxygen consumption in larvae. The left and right peaks represent
 191 standard metabolic rate SMR and routine metabolic rate RMR, respectively
 192

193 *Cardiac frequency*

194
 195 Experimental protocol

196 In order to assess cardiac frequency f_H , 101 other larvae F_1 were tested individually ($n_{control} = 22$, $n_{0.3X} = 30$, $n_{1X} =$
 197 23 , $n_{3X} = 26$). The low pigmentation and transparency of larvae at 5dpf permitted to visually measure f_H through
 198 the body wall. In order to do so, individuals were placed into 2% agar groove on petri dishes (*i.e.* 6-7 ml). The
 199 addition of 3% methylcellulose permitted to maintain larvae motionless in lateral position. Previous tests
 200 permitted to determine that two hours-acclimation were necessary to distress from the transfer and reach basal
 201 heart rate. Three consecutive videos of 30 seconds were recorded to observe *in vivo* beating heart of each larva.
 202 The use of a microscope (Olympus SZX9), above which was placed a color camera (DMK 31AU03- The
 203 Imaging sources, Germany), permitted to record videos on IC Capture software at 15 images per second.

204
 205 Videos analysis

206 Estimation of f_H was based on the principle that each passage of blood from the atrium to the ventricle
 207 corresponds to a cardiac contraction. In transparent larvae, blood circulation between these two cardiac chambers
 208 induces a difference in contrast on which the video analysis will be based. To improve grey contrasts, Virtual
 209 Dub software was used to convert the video in black and white through the addition of filter. Videos were
 210 recorded in bitmap format. As each movie lasted 30s and was recorded at 15 images per second, around 450
 211 images were extracted for each video. Each of these sequences of images was then imported in the software
 212 Image J, where the use of the Plugin *ROI* (*i.e.* Region of interest) permitted to automatically delimit the atrium
 213 and the ventricle, and to calculate the alternate circulation between these two areas of interest. Data obtained
 214 were exported in Statistica for Fourier analysis. This analysis allows assessment of cardiac frequency from the
 215 periodical movement of the heart contraction.

216
 217

218 *Cardiac mRNA expression*

219

220 Transcript abundance of some genes encoding key proteins in cardiac development and activity (cardiac myosin
 221 light chain 1 (*cmlc1*), cardiac troponin type T2A (*tnnt2a*) and calcium transporting ATPase (*atp2a2a*) was
 222 examined in embryo (48hpf) whose genitors were exposed to 1X and control treatments and larvae F₁ (6dpf)
 223 from genitors exposed to 0.3X, 1X and control treatments.

224

225 Preparation of total RNA

226 Fish were euthanized and frozen on liquid nitrogen prior to storage at -80°C. Total RNA from pools of 30
 227 embryo (48hpf) or 15 larvae F₁ (6dpf) was extracted using TRIzol (Invitrogen Ltd, Cergy Pontoise, France). Five
 228 to six pools were used per treatment (0.3X, 1X and *control*). Due to a limited number of embryos from genitors
 229 exposed to 3X treatment, it was not possible to measure mRNA expression in these descendants. RNA was
 230 quantified by measuring the optical density at 260 nm (OD₂₆₀). RNA integrity was checked using the
 231 Bioanalyzer 2100 Agilent and RNA 6000 LabChip® kits (Agilent Technologies, Waldbronn, Germany)
 232 according to the manufacturer's instructions

233

234 Real time RT-PCR

235 For cDNA synthesis, 1.5 µg of RNA was denatured in the presence of 0.5 µg random primers/µg RNA (Promega)
 236 for 5 min at 70°C, and then chilled on ice. Reverse transcription (RT) was performed at 37°C for 1 h using M-
 237 MLV reverse transcriptase (Promega), as described by the manufacturer. To inactivate the reverse transcription,
 238 samples were heated at 95°C for 10 min and then stored at -20°C. The final volume for cDNA sample was 25µl.
 239 Control reactions were run without reverse transcriptase and used as negative control in the real-time PCR study.
 240 Real-time RT-PCR was carried out on a StepOnePlus real time PCR system (Applied Biosystem, Courtaboeuf,
 241 France). Reactions were performed in a total volume of 10µl with 4µl diluted RT reaction, 5µl Fast SYBR-
 242 Green master Mix (Applied Biosystem, Courtaboeuf, France) and 300nM of each primer. Primer pairs are given
 243 in Table 2. A melting curve was generated to confirm product specificity. Expression levels of target genes were
 244 normalized to the geometric average of two housekeeping genes (β-actin and ribosomal protein, L13a *rpl13a*).
 245 Expression data were calculated by the 2^{-ΔΔCt} method.

246

Table 2: Primer pairs for real time quantitative RT-PCR

gene name	Forward primer sequences	Reverse primer sequences	references
<i>cmlc1</i>	CTCCACACTGGGCATACCTT	CTGCACCAGTTCCAGAGACA	Nesan and Vijayan 2012
<i>tnnt2a</i>	CATCCAGCTCCACAATTCCT	GGCGGTTACATGCAAAAGAT	Nesan and Vijayan 2012
<i>atp2a2a</i>	GCAGGTTAGAGCCGTTTCTG	CTGTGCCTTGTGCAATGACT	Nesan and Vijayan 2012
<i>β-actin</i>	TGTTTTCCCTCCATTGTTGGAC	CGTGCTCAATGGGGTATTTGAGG	Saito et al 2010
<i>rpl13a</i>	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	Tang et al 2007

247

248

249

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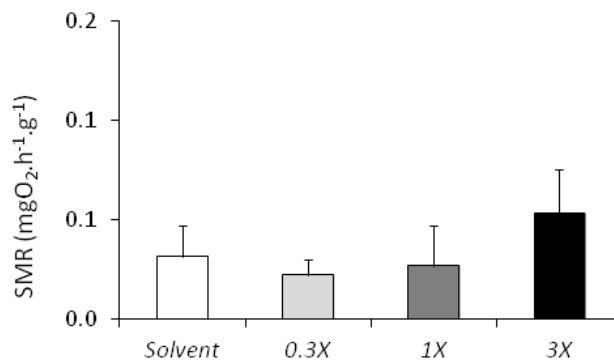
251 *Statistical analysis*

252
253 The statistical analysis was carried out using Statistica software. For analyses regarding aerobic metabolism and
254 cardiac frequency variables, the normality and homoscedaticity were checked through the Shapiro and Barlett
255 tests, respectively. These conditions, necessary to apply parametric tests, were respected. A test of ANOVA was
256 therefore used to determine significant differences due to the treatment (0.3X, 1X, 3X, control). If necessary, a
257 Tuckey post-hoc test was applied to determine which treatments differed significantly from the control. The
258 results were considered to be significantly different when $p < 0.05$. Regarding cardiac mRNA expression,
259 difference between groups and comparison of two means were assessed using Kruskal–Wallis one-way analysis
260 of variance and Mann-Whitney U test. For all, significance was accepted at a level of 0.05.

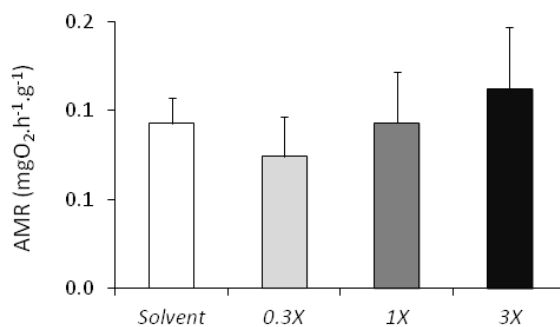
261
262 **Results**

263 *Active metabolic rate, Standard metabolic rate, Aerobic metabolic scope*

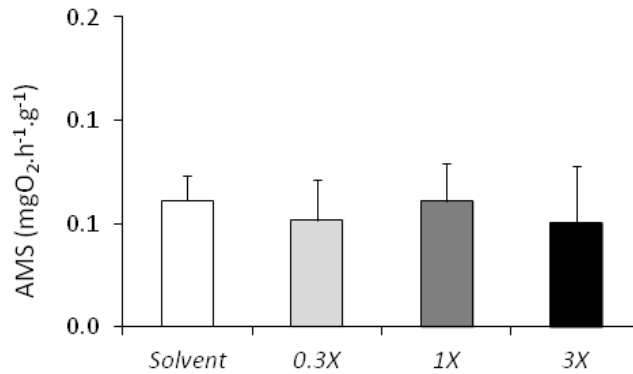
264 Statistical analyses did not reveal any significant difference in AMR (Fig. 2A, Anova, $ddl=3$, $F=2.46$, $p=0.08$)
265 and AMS (Fig. 2C, Anova, $ddl=3$, $F=0.32$, $p=0.81$) measured in 5dpf larvae born from genitors exposed to one of
266 the 4 treatments tested (i.e. control, 0.3X, 1X and 3X). On the other hand, the treatment had a significant effect on
267 SMR (Fig. 2B, Anova, $ddl=3$, $F=32.61$, $p < 0.01$). However, post-hoc test revealed that none of the three
268 treatments based on contaminated diet (i.e. 0.3X, 1X, 3X) differ significantly from the control (Tuckey test
269 $p > 0.05$ in each case). Significant differences were only observed between larvae from genitors exposed to the 3X
270 treatment and both of the other PAH treatments (i.e. 0.3X, 1X; Tuckey test $p_{0.3X-3X} < 0.05$, $p_{1X-3X} < 0.05$).
271
272



273



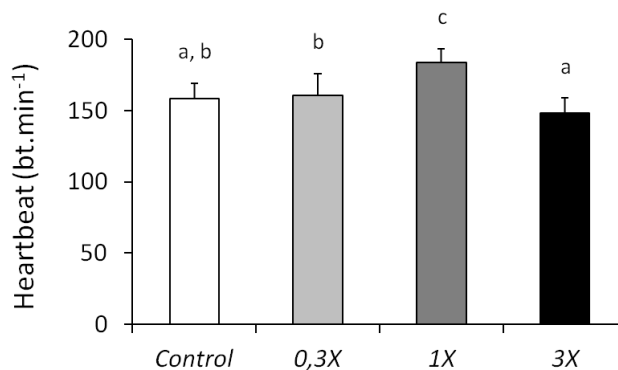
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275
 276 Fig. 2 Active metabolic rate (AMR, A), standard metabolic rate (SMR, B), and aerobic metabolic scope (AMS, C)
 277 of 5 dpf larvae F1 zebrafish *Danio rerio* coming from genitors exposed to one of the following treatments:
 278 control, 0.3X, 1X, and 3X. The environmental reference concentration X was 5.5 µg.g⁻¹ of dry food. For SMR
 279 (B), treatment with common superscript is not significantly different
 280

281 *Cardiac frequency*

282
 283 PAHs exposure induced a significant effect on the cardiac frequency f_H measured in the 5dpf larvae F₁ (Fig 3;
 284 ANOVA, ddl=3, F=32.61, p<0.01). Post hoc test revealed that only cardiac frequency of larvae born from 1X
 285 genitors differed significantly from *control* larvae with in average $f_H = 183.8 \pm 9.7$ bt.min⁻¹ (Tuckey test, p<0.01).
 286

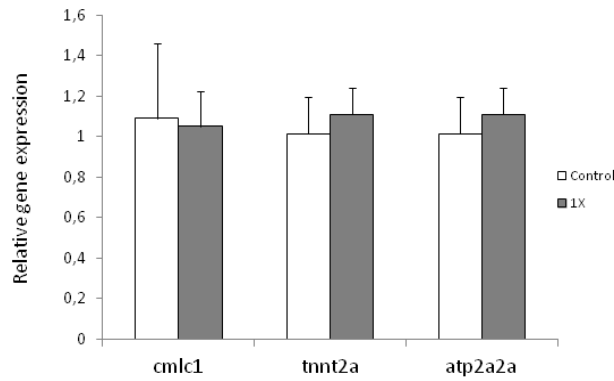


287
 288 Fig. 3 Cardiac frequency of 5 dpf larvae F1 zebrafish *Danio rerio* coming from genitors exposed to one of the
 289 following treatments: control, 0.3X, 1X, and 3X. The environmental reference concentration X was 5.5 µg.g⁻¹
 290 of dry food. Mean values±standard deviation are plotted and treatments with common superscript are not
 291 significantly different
 292

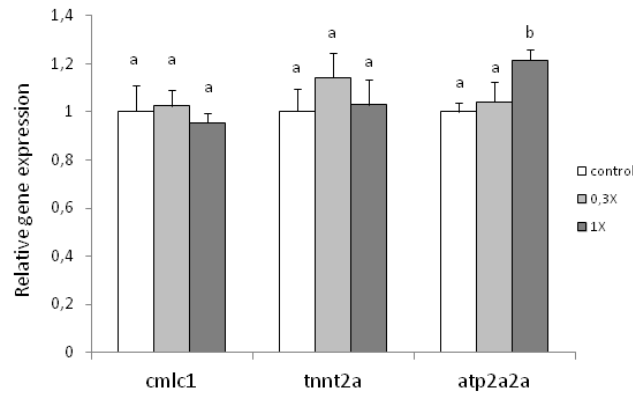
293
 294 *Cardiac mRNA expression*

295
 296 The mRNA abundance of cardiac muscle genes measured in 48hpf embryo F₁ was similar between embryo from
 297 genitors exposed to 1X pyrolytic PAHs or control (Fig. 4). In 6dpf larvae F₁, mRNA expression for myosin light
 298 chain 1 and troponin type T2A was similar in larvae F₁ whatever the diet the genitors were exposed to (Fig 5).
 299 The mRNA expression of calcium transporting ATPase *atp2a2a* gene showed a different pattern. This gene was
 300 indeed upregulated in larvae F₁ whose genitors were exposed to 1X pyrolytic PAHs (Mann-Whithney U test,
 301 p<0.01).

302
303



304
305 Fig. 4 Relative mRNA expression of three cardiac structural genes in 48 hpf embryo F1 zebrafish *Danio rerio*
306 from genitors exposed to pyrolytic PAHs 1X treatment. Mean values \pm standard deviation are plotted (N=6 pools
307 of 30 embryo for control and N=5 for 1X treatment)



308
309 Fig. 5 Relative mRNA expression of three cardiac structural genes in 6 dpf larvae F1 zebrafish *Danio rerio*
310 from genitors exposed to pyrolytic PAHs 0.3X and 1X treatment. The environmental reference concentration X was
311 $5.5 \mu\text{g}\cdot\text{g}^{-1}$ of dry food. Mean values \pm standard deviation are plotted (N=5 pools of 15 larvae for each treatment).
312 For each gene, treatments with common superscript are not significantly different
313

314
315 **Discussion**
316

317 This study carried out in zebrafish, assessed the effects of parental exposure to pyrolytic PAHs mixtures on
318 biological parameters related to the metabolic and cardiac performance of their progeny. Regarding cardiac
319 performance, heart rate and mRNA expression of genes encoding for cardiac activity were both modified at the
320 environmental representative PAHs concentration 1X. On the other hand, the unique metabolic variable
321 influenced was the SMR, but not in comparison to the control treatment.

322
323 Cardiac frequencies measured (i.e. $158.2 \pm 10.7 \text{ bt}\cdot\text{min}^{-1}$) for the controls are consistent with previous
324 investigations on zebrafish. Barrionuevo and Burggren (1999) and Burggren (2005) estimated average cardiac
325 frequency to be $155 \text{ bt}\cdot\text{min}^{-1}$ in 5dpf larvae. The present results showed a 12.5% significant increase in cardiac
326 frequency of larvae born from genitors exposed to the 1X PAHs treatment (Fig. 3). In addition, calcium

327 transporting ATPase mRNA, a key cardiac gene essential for contraction (Ebert et al. 2005), was observed to
328 increase significantly for the same type of contamination (Fig 5). This suggests that in the progeny whose parents
329 were exposed to the PAHs concentration 1X, heart presents an abnormal tachycardia pattern.

330

331 On the contrary, complementary work showed a similar mRNA expression of cardiac structural gene (cardiac
332 myosin light chain 1 (*cmlc1*) and cardiac troponin type T2A (*tnnt2a*) in the progeny of parents exposed or not to
333 PAHs treatments. This suggests a correct cardiac morphogenesis as it was observed on larvae used for cardiac
334 frequency measurements. The pattern observed in the present study differs from recent studies investigated
335 cardiotoxicity of PAHs in early life-stages of zebrafish directly exposed to PAHs by sediment contacts or oil.
336 Cardiac defects observed were indeed principally heart deformities (e.g. pericardial oedema), arrhythmia or
337 bradycardia, and never tachycardia (Incardona et al. 2003, 2004, 2005, 2006, 2011; Hicken et al. 2011; Perrichon,
338 personal communication). However, comparison is limited since (i) most of these investigations have tested
339 individual compounds at unrealistic environmental concentrations in order to trigger toxic mechanisms that
340 impair physiological or metabolic functions (Incardona et al. 2005, 2006, 2011) and (ii) fish were directly
341 exposed to pollutants whereas in the present study contamination was expected to occur by parental transfer.
342 Moreover, depending on the way of exposure, PAHs levels of contamination in organisms are expected to be
343 different. In the present study, PAHs-metabolites concentrations measured in larvae F₁ tended to be proportional
344 to the PAHs degree of exposure but the levels of contamination are low. Focusing on fish directly exposed,
345 PAHs-metabolites have not been assessed in genitors at 4 months but Vignet et al., (in press) measured it on 15
346 dpf larvae. The concentrations tended to be proportional to the PAHs content of the food they received 9.1 ± 0.62 ,
347 19.3 ± 2.91 , 72 ± 11.79 , 275.11 ± 50.14 for *control*, 0.3X, 1X, 3X respectively. These differences in PAHs-
348 metabolites concentrations may be explained by the fish capacity to quickly metabolize PAHs in more water-
349 soluble components easier to excrete (Blahova et al., 2013, Dévier et al., 2013; Tuvikene, 1995). Probably only a
350 small part of these hydrocarbons derivatives has been accumulated in genitor's gonads and transferred into the
351 eggs of zebrafish, as shown in several studies in aquatic animals (Lotufo, 1998; Miller, 1993; Monteverdi and
352 Giulio, 2000; Hall and Oris, 1991). The lack of information regarding PAHs parental transfer in literature and
353 these PAHs-metabolites concentrations may participate to the fact that no clear pattern regarding the effect of
354 parental exposure to PAHs on the progeny cardiac frequency can be established.

355 Another hypothesis to explain the observed tachycardia pattern is that PAHs exposure could have indirect effects
356 on cardiac activity through production of stress hormones related to the pollutant exposure, as observed in other
357 studies (e.g. *Oncorhynchus mykiss*, Aldegunde et al., 1999; Tintos et al., 2008; *Clupea pallasii*, Kennedy and
358 Farrell, 2005; *Neogobius melanostomus*, Marentette et al., 2013). In the present study, the cortisol level was not
359 measured. However, as observed in other species, *D. rerio* is likely to present an elevation of the production of
360 stress hormone induced by the exposure to pyrolytic PAHs. This cortisol increase could modulate cardiac
361 frequency (e.g. bradycardia or tachycardia, McCormick and Nechaev, 2002; Nesan & Vijayan, 2012). A
362 relevant properties of the maternal cortisol, produced under stressor, is that it could be accumulated in eggs,
363 affect the gametes quality (Campbell et al., 1992, 1994), the size or the quality of the progeny of fish
364 (McCormick 1998, 1999, 2006). Based on the cortisol properties, we can suppose that this stress hormone could
365 modulate cardiac activity and induce tachycardia in the present study. Moreover our results could be correlated
366 with fish hyperactivity and the high level of stress, observed during behavioral challenge in other similar study

367 (Perrichon, personal communication).

368

369 Aerobic metabolism of the progeny coming from genitors directly exposed to PAHs never differed significantly
370 compared to control; even for the treatment *IX* for which cardiac frequency was significantly influenced. Very
371 few studies have examined influence of PAHs exposure on cardio-respiratory performance (e.g. Claireaux and
372 Davoodi, 2010, Hicken 2011). Associated to a decrease in cardiac performance, Claireaux and Davoodi (2010)
373 have observed that fuel-exposed common sole displayed lower aerobic metabolism compared to control. Hicken
374 et al. (2011) also demonstrated that early exposure to crude oil altered cardiac morphology (by modifying
375 ventricular shape), which reduced cardio-respiratory performance and aerobic capacity in adult zebrafish. On the
376 contrary, in the present study, variations in aerobic metabolism and cardiac frequency did not match in the larvae
377 tested (Fig. 2 and 3). This may have different explanations. First, it is worth noticing that cardiac frequency is
378 only one cardiac parameter on which depends the oxygen transport. Taking into consideration other parameters,
379 such as the stroke volume, should give a better overview of an organism cardiac performances. Second, the
380 proportion of cutaneous respiration is expected to be high in early life stage, which can distort the oxygen
381 transport capacity. Third, larvae were tested in group of 10 individuals, while cardiac frequency was measured in
382 individuals. All these reasons may also count for the different results observed later in 2-months juveniles from
383 the same generation F_1 for which it was observed a significant decrease in both *AMR* and *AMS* in the treatment
384 *3X* (Lucas et al., *submitted*). These juveniles were indeed individually tested, which allowed more acute
385 measurements of metabolic rates.

386

387 **Conclusion**

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390 The lack of notably impairments we observed does not imply that PAHs effects were absent but that the level of
391 integration is probably finer at larval stage. Investigation on heart morphology could provide complementary
392 information regarding these results. For instance, as these capacities depend on cardiac form and function, it
393 could be interesting to observed atrial and ventricular shape in 5dpf larvae through specific antibodies marking
394 on these structures. Moreover, as suggested by few studies, maternal transfer of pollutant to fish eggs and larvae
395 could acclimatize them to toxicants, inducing potential compensations at lower level of organization (Peake et al.
396 2004, Nye et al. 2007). In addition, it is worth noticing that cardiac frequency and metabolism was estimated in
397 individuals, while PAHs and metabolites dosages were made on larval pools. This could participate to a
398 mismatch in the variability related these two variables.

399

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643 **Figures captions**
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645 **Figure 1.** An example of frequency distribution of oxygen consumption in larvae. The left and right peaks
646 represent *standard metabolic rate SMR* and routine metabolic rate *RMR*, respectively.

647 **Figure 2.** Active metabolic rate (*AMR, A*), standard metabolic rate (*SMR, B*) and aerobic metabolic scope (*AMS,*
648 *C*) of 5dpf larvae F₁ zebrafish *Danio rerio* coming from genitors exposed to one of the following treatments:
649 control, 0.3*X*, 1*X* and 3*X*. The environmental reference concentration *X* was 5.5 µg.g⁻¹ of dry food. For *SMR* (*B*),
650 treatment with common superscript are not significantly different.

651 **Figure 3.** Cardiac frequency of of 5dpf larvae F₁ zebrafish *Danio rerio* coming from genitors exposed to one of
652 the following treatments: control, 0.3*X*, 1*X* and 3*X*. **The environmental reference concentration *X* was 5.5**
653 **µg.g⁻¹ of dry food.** Mean values ± standard deviation are plotted and treatments with common superscript are not
654 significantly different.

655
656 **Figure 4.** Relative mRNA expression of three cardiac structural genes in 48hpf embryo F₁ zebrafish *Danio rerio*
657 from genitors exposed to pyrolytic PAHs *1X* treatment. Mean values ± standard deviation are plotted (N=6 pools
658 of 30 embryo for control and N=5 for 1*X* treatment).

659
660 **Figure 5.** Relative mRNA expression of three cardiac structural genes in 6dpf larvae F₁ zebrafish *Danio rerio*
661 from genitors exposed to pyrolytic PAHs 0.3*X* and 1*X* treatment. **The environmental reference concentration**
662 ***X* was 5.5 µg.g⁻¹ of dry food.** Mean values ± standard deviation are plotted (N=5 pools of 15 larvae for each
663 treatment). For each gene, treatments with common superscript are not significantly different.

664