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## Trophic contamination by pyrolytic polycyclic aromatic hydrocarbons does not affect aerobic metabolic scope in zebrafish *Danio rerio*

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### ► To cite this version:

Julie Lucas, Antoine Bonnieux, Laura Lyphout, Xavier Cousin, P Miramand, et al.. Trophic contamination by pyrolytic polycyclic aromatic hydrocarbons does not affect aerobic metabolic scope in zebrafish *Danio rerio*. *Journal of Fish Biology*, 2016, 88 (1), pp.433-442. 10.1111/jfb.12835 . hal-01451297

**HAL Id: hal-01451297**

**<https://hal.science/hal-01451297>**

Submitted on 31 Jan 2017

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1 **Trophic contamination by pyrolytic polycyclic aromatic hydrocarbons**  
2 **does not affect aerobic metabolic scope in zebrafish *Danio rerio***

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24 **Abstract**

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26 The effect of trophic exposure to pyrolytic polycyclic aromatic hydrocarbons (PAH) on  
27 aerobic metabolism on zebrafish *Danio rerio* was investigated. There were no significant  
28 differences in standard metabolic rate (*SMR*), active metabolic rate (*AMR*) or aerobic  
29 metabolic scope (*AS*) at any sub-lethal concentration of PAH in the diet of adult or  
30 juvenile zebrafish. This suggests that under these experimental conditions, exposure to  
31 PAH in food did not influence aerobic metabolism of this species.

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33 Keywords: metabolic rates, static respirometry, sub-lethal concentration, petroleum  
34 hydrocarbons

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46 Present as complex mixtures in environment, pyrolytic polycyclic aromatic hydrocarbons  
47 (PY PAH) result from combustion of organic matter and enter aquatic ecosystems  
48 through atmospheric deposition (Hylland, 2006). Due to their high liposolubility, PAH are  
49 typically adsorbed by organic matter or marine sediments, bioaccumulated by organisms  
50 at the lowest trophic levels (e.g. invertebrates; O'Connor & Lauenstein, 2006; Bustamante  
51 *et al.*, 2012) and transferred through trophic chains (Hylland, 2006; Vignet *et al.*, 2014a,  
52 2014b).

53 In the context of environmental changes and risk management, assessment of the  
54 toxicity of PAH is necessary to evaluate their impacts on aquatic life. Past studies on  
55 fish have demonstrated that hydrocarbons have carcinogenic (Hawkins *et al.*, 1990;  
56 Myers *et al.*, 1991; Larcher *et al.*, 2014), genotoxic (e.g. DNA damage; Holth *et al.*,  
57 2008; Nogueira *et al.*, 2009) and ontogenic effects (Hornig *et al.*, 2010; Incardona *et al.*,  
58 2011; Singh *et al.*, 2008). They have also been found to affect reproduction (Collier *et al.*,  
59 1992; Seruto *et al.*, 2005; Vignet *et al.* 2014a), growth (Meador *et al.*, 2006; Kim *et al.*,  
60 2008, Vignet *et al.*, 2014a), metabolism (Wedemeyer & McLeay, 1984; Wedemeyer *et al.*,  
61 1990; Davoodi & Claireaux, 2007;) and behaviour (Gonzales-Doncelet *et al.*,  
62 2008; Vignet *et al.*, 2014b).

63 This study aimed at investigating responses of fish exposed to PAH through the  
64 assessment of aerobic metabolic scope (AS) as an indicator of the physiological state of  
65 the organism (Fry, 1947). AS represents the amount of oxygen the animal is able to  
66 provide for all activities beyond standard metabolism (e.g. locomotion, digestion,  
67 feeding; Fry, 1947, 1971). It is defined as the difference between active metabolic rate  
68 (AMR), which is the highest metabolic rate the organism can sustain, usually during

69 maximal activity, and the standard metabolic rate (*SMR*), the metabolic rate necessary to  
70 maintain vital functions and measured under resting conditions at a known ambient  
71 temperature (e.g. Brett, 1964; Fry, 1947; White *et al.* 2006). *AS* is known to be modulated  
72 by pollutants (e.g. Sharp *et al.*, 1979; Hose & Puffer, 1984; Correa & Garcia, 1990;  
73 Davison *et al.*, 1992; Nikinmaa, 1992; Wilson *et al.*, 1994; Lannig *et al.*, 2006; Johansen  
74 & Jones, 2011; Davoodi & Claireaux, 2007; Christiansen *et al.*, 2010). Focussing on  
75 PAH, Davison *et al.* (1992) reported that the Antarctic fish *Pagothenia*  
76 *borchgrevinki* (Boulenger, 1902) doubled its *AS* after exposure to an aqueous fraction of  
77 petroleum. Davoodi & Claireaux (2007) showed a 30% decrease of *AS* in the common  
78 sole *Solea solea* (Quensel, 1806) acutely exposed to a fuel. This *AS* reduction could be  
79 explained by malfunctions in organs involved in oxygen transport (e.g. heart, gills) and  
80 an associated decrease of *AMR* (Claireaux, 2004). Another possibility to reduce *AS* is the  
81 setting up of supplementary energy-demanding detoxification processes, which could  
82 increase *SMR* (Lannig, 2006).

83 This study aimed to determine the impacts of environmentally relevant  
84 concentrations of PY PAH on the aerobic metabolism of zebrafish *Danio rerio* (F.  
85 Hamilton, 1822) contaminated by ingestion. The main hypothesis was that chronic  
86 exposure to PY PAH would impair *AS* by increasing *SMR* and/or reducing *AMR*. The  
87 consequent potential reduction of *AS* would indicate a decrease in the capacity of the fish to  
88 support oxygen-demanding activities beyond *SMR*. Metabolic variables were assessed for  
89 two durations of chronic exposure, 2 and 6 months (juvenile and adult stages,  
90 respectively).

91 Pairof *D. rerio* (wild-type Tuebingen strain) were reared together in 10 ltanks.  
92 Aquaria were filled with water prepared as a mixture of reverse osmosis-treated water  
93 and tap water, both filtered through sediment and activated charcoal filters. Rearing  
94 conditions were: temperature  $28 \pm 0.5$  °C, conductivity  $300 \pm 50$   $\mu\text{Scm}^{-1}$ , air saturation  
95  $\geq 80\%$ , pH  $7.5 \pm 0.5$ , photoperiod of 14 h light/10 h dark. The fish were fed twice daily  
96 with commercial dry food (INICIO Plus, BioMar, [www.biomar.com](http://www.biomar.com)), occasionally  
97 supplemented with red sludge worms (Boschetto-Frozen fish food,[www.achat-](http://www.achat-aquarium.fr)  
98 [aquarium.fr](http://www.achat-aquarium.fr)). Over a period of one month, spawn was obtained weekly from pairs  
99 following the protocol described in Lucas *et al.* (2014a) and Vignet *et al.* (2014a).Spawn  
100 was then mixed to avoid any parental influence.

101 At two weeks old, fish were kept in groups of 30 individuals in 10 l aquaria.  
102 Contamination with PY PAH was achieved through the trophic pathway. Artificial dry  
103 food was contaminated with a mixture of PYPAH. The mixture was composed of 95%  
104 non-substituted PAH with a majority of four- and five-ring PAH; a detailed description is  
105 given in Vignet *et al.* (2014a). The PAH concentration targeted for contamination of  
106 pellets was  $5000$   $\text{ngg}^{-1}$ , based on concentrations measured in molluscs in the Seine  
107 estuary. This reference environmental concentration is hereafter referred to as  $X$ ; it  
108 represents one of the four treatments tested in this study (PAH concentrations measured  
109 in diet  $[\text{PAH}] = 5816 \pm 1433$   $\text{ngg}^{-1}$ ). Based on this reference, two other treatments were  
110 tested: a lower concentration of  $0.3X$  ( $[\text{PAH}] = 1763 \pm 468$   $\text{ngg}^{-1}$ ) and a higher  
111 concentration of  $3X$  ( $[\text{PAH}] = 18151 \pm 4983$   $\text{ngg}^{-1}$ ). A fourth (control) treatment was  
112 added in which dry food was exposed only to dichloromethane, the solvent used to carry  
113 the PAH. Contamination through food was achieved by feeding fish twice a day with one

114 of the four treatments. Quantification of hydroxylated metabolites in larvae at 15 days post  
 115 fertilisation (dpf) indicated a dose-dependent increase of metabolites  
 116 confirming successful contamination (total concentrations of hydroxylated metabolites for  
 117 each treatment: control = 9.1 ngg<sup>-1</sup> of tissue; 0.3X = 20 ngg<sup>-1</sup>, 1X = 72 ngg<sup>-1</sup>; 3X = 275  
 118 ngg<sup>-1</sup>; Vignet *et al.*, 2014a). Fish were fed with treated pellets from their first meal (5 dpf)  
 119 to the ages of 2 months for juveniles and 6 months for adults (Table 1) with size-adapted  
 120 food ( $\leq 125\mu\text{m}$ , 125–315 $\mu\text{m}$ , 315–500 $\mu\text{m}$ ,  $\geq 500\mu\text{m}$ ). In accordance with protocols  
 121 in Vignet *et al.* (2014a), larvae were fed *ad libitum* and then, starting from two months  
 122 old, the ration of food was 5 % to 2% of the biomass in each tank in order to maintain  
 123 constant growth. For all fish, brine shrimps (Ocean Nutrition Europe BVBA,  
 124 <http://www.oceannutrition.eu/fr/default.aspx>) were given as supplementary food once a  
 125 day. Characteristics of the fish are reported Table 1.

126 **Table I.** Biometry of juveniles and adults *Danio rerio* in each treatment (mean  $\pm$  SE). *X* is  
 127 the environmental reference concentration of 5.5  $\mu\text{g PY PAHs.g}^{-1}$  of dry food. Control  
 128 was food that had been exposed to dichloromethane only and did not contain PY PAHs.  
 129

Treatment	Lifestage	Number of fish (n)	Weight (g)	Standard length (cm)	Total length (cm)
<i>Control</i>	Juveniles	24	0.20 $\pm$ 0.08	2.23 $\pm$ 0.29	2.72 $\pm$ 0.37
	Adults	15	0.63 $\pm$ 0.19	3.08 $\pm$ 0.28	3.80 $\pm$ 0.35
<i>0.3X</i>	Juveniles	23	0.21 $\pm$ 0.12	2.18 $\pm$ 0.27	2.73 $\pm$ 0.20
	Adults	12	0.68 $\pm$ 0.11	3.26 $\pm$ 0.20	3.94 $\pm$ 0.20
<i>1X</i>	Juveniles	24	0.178 $\pm$ 0.04	2.21 $\pm$ 0.08	2.69 $\pm$ 0.15
	Adults	15	0.57 $\pm$ 0.13	3.00 $\pm$ 0.32	3.69 $\pm$ 0.30
<i>3X</i>	Juveniles	24	0.18 $\pm$ 0.08	2.18 $\pm$ 0.29	2.65 $\pm$ 0.33
	Adults	15	0.44 $\pm$ 0.16	2.84 $\pm$ 0.40	3.36 $\pm$ 0.41

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133 To assess the aerobic metabolic rate of fish, eight identical circular size-adapted  
134 respirometers (diameter: 3.75 cm, volume: 0.061 l for juveniles and 7.50 cm, 0.179 l for  
135 adults) were employed. These were immersed in two buffer tanks (depth x length x height:  
136 10 x 75 x 75 cm for both juveniles and adults) filled with temperature-controlled  
137 and aerated water. Oxygen consumption was measured by intermittent-flow respirometry  
138 (Steffensen, 1989) where the water supply in each respirometer was provided by flush  
139 pumps controlled by a timer. This system alternated phases of flushing and oxygen  
140 renewal with phases of measurement of oxygen consumption ( $MO_2$ ), each of which  
141 lasted 30 min. Finally, a multichannel peristaltic pump was installed to create continuous  
142 water flow and ensure water mixing inside each of the chambers. Each respirometer was  
143 equipped with an optic fibre sensor (PreSens, [www.presens.com](http://www.presens.com)) connected to a  
144 multichannel oxygen measuring system (OXY 4 mini, PreSens) to record the level of  
145 dissolved oxygen in the water. Oxygen data was sampled each five seconds with the  
146 program Oxyview (PreSens).

147 Fish were starved 24 h prior to respirometry. For each trial, eight fish (two in each  
148 treatment) were tested individually in respirometer, in two consecutive phases. First, to  
149 increase fish metabolism and assess *AMR*, each fish was transferred and chased with a  
150 stick in a 1 l tank (Schurmann & Steffensen, 1997; Lefrançois & Claireaux, 2003; Jourdan-  
151 Pineau *et al.*, 2010; Clark *et al.*, 2012; Cannas *et al.*, 2013). When the fish was  
152 fatigued (did not respond to the stimulation), it was transferred into a respirometer. The  
153 oxygen consumption of the fish was immediately recorded for 30 min to  
154 calculate *AMR*. Then, to confirm the accuracy of the *AMR* assessment, each fish was  
155 chased again in the respirometer and its  $MO_2$  measured again for a new period of 30 min.

156 The second step consisted of a resting period of 48 h to reach and estimate *SMR*.  
157 During this period fish were undisturbed and  $MO_2$  was regularly and automatically  
158 measured. From these oxygen measurements, *SMR* was estimated according to the  
159 method described by Steffensen *et al.* (1994). Briefly, the frequency distribution of  $MO_2$   
160 values recorded during the last 24 h of the test was plotted. This generally produces a  
161 bimodal frequency distribution due to the routine activity of the fish. The higher mode  
162 (the first peak) is considered to reflect *SMR* and the lower mode (the second peak)  
163 corresponds to the routine metabolic rate (*RMR*), the energy required by the fish for  
164 maintenance plus random activity. During all the experiments, oxygen concentration  
165 was never lower than 75% oxygen saturation in each respirometer.

166 After the 48 h period of resting  $MO_2$  measurements, fish were removed from  
167 the respirometers and anaesthetised using benzocaine (50 mg l<sup>-1</sup>). The length (standard and  
168 total length) and mass of each individual were determined (Table 1). To quantify  
169 microbial oxygen consumption in the respirometer, a blank measurement was carried out  
170 before and after each trial. A linear change in background  $MO_2$  over the 48 h experimental  
171 trial was assumed and subtracted the expected value from the corresponding total  $MO_2$   
172 measured.

173 Oxygen consumption ( $MO_2$ ), expressed in mg O<sub>2</sub>g<sup>-1</sup>h<sup>-1</sup>, was calculated according  
174 to the formula  $MO_{2meas} = \Delta[O_2] V \Delta t^{-1} M_{meas}^{-1}$ , where  $\Delta[O_2]$  (in mg O<sub>2</sub> l<sup>-1</sup>) is the change in  
175 oxygen concentration during the measurement period  $\Delta t$  (in h),  $V$  (in l) is the volume of the  
176 respirometer minus the volume of the fish, and  $M_{meas}$  (in g) is the measured mass of  
177 the fish. An allometric relationship between oxygen consumption and body mass, allows  
178 correction of  $MO_{2meas}$  using the formula  $MO_{2cor} = MO_{2meas} (M_{meas} M_{cor}^{-1})^{1-b}$ , where  $MO_{2cor}$  (in

179  $\text{mg O}_2\text{g}^{-1}\text{h}^{-1}$ ) is the oxygen consumption related to a standard fish of 1 g ( $M_{cor}$ ),  $MO_{2meas}$   
180 (in  $\text{mg O}_2\text{g}^{-1}\text{h}^{-1}$ ) is the oxygen consumption estimated for experimented fish whose mass  
181 was  $M_{meas}$  (in g) and  $b$  is the allometric scaling exponent describing the relationship  
182 between oxygen consumption and body mass of fish. In previous study,  $b$  was found to be  
183 equal to 0.926 and 0.965 in the case of *AMR* and *SMR* assessment respectively (Lucas *et*  
184 *al.*, 2014a). Aerobic metabolic scope (*AS*) was calculated as the difference between *AMR*  
185 and *SMR*. *AMR*, *SMR* and *AS* were assessed once for each individual.

186 Statistical analysis was carried out using Graphpad Prism software. As the  
187 conditions of normality (tested using the Kolmogorov–Smirnov test) and  
188 homoscedasticity (tested using the Bartlett test) of data were not met, a Kruskal–Wallis  
189 non-parametric test was used to test for significant differences in metabolic rates among  
190 treatments. If necessary, a Dunn post-hoc test was applied to determine which treatments  
191 differed significantly. Differences were considered to be significant when  $P < 0.05$ .

192 There were no significant differences in *AMR*, among treatments for either  
193 juveniles or adults ( $P = 0.45$  and  $P = 0.93$  for juveniles and adults, respectively; Fig. 1A).  
194 Similarly there were no significant differences in *SMR* among treatments for each life  
195 stage ( $P = 0.23$  and  $P = 0.74$  for juveniles and adults, respectively; Fig. 1B).  
196 Therefore, *AS* also did not differ significantly among the treatments for juveniles ( $P = 0.59$ )  
197 or adults ( $P = 0.89$ ) (Fig. 1C).

198 This is the first study assessing the aerobic metabolism of zebrafish chronically  
199 exposed to a mixture of pyrolytic PAH. Under these experimental conditions and at the  
200 two life stages tested, trophic exposure to PY PAH did not affect *SMR*, *AMR* or *AS* of this  
201 species. It is worth noting that these data for aerobic metabolism (Fig. 1B) are in

202 agreement with previous studies carried out for the same species and with a similar  
 203 experimental approach ( $SMR = 0.19 \text{ mg O}_2\text{g}^{-1}\text{h}^{-1}$  in Barrionuevo & Burggren, 1999;  
 204  $SMR = 0.31 \pm 0.11 \text{ mg O}_2\text{g}^{-1}\text{h}^{-1}$  and  $0.35 \pm 0.16 \text{ mg O}_2\text{g}^{-1}\text{h}^{-1}$  in juveniles and adults,  
 205 respectively, in Lucas *et al.*, 2014a).

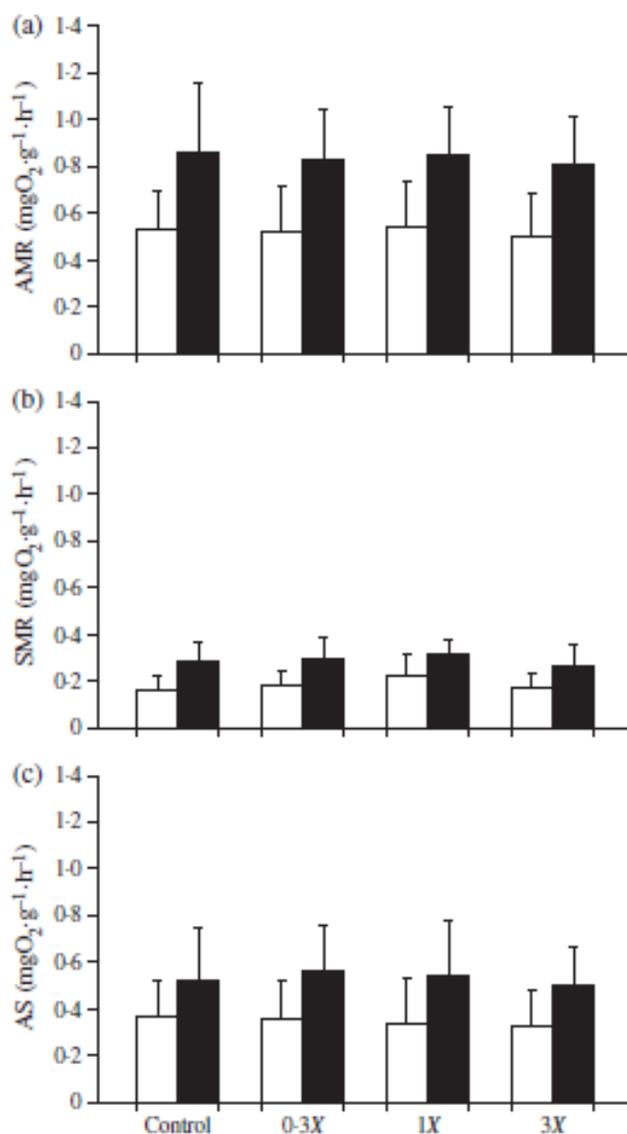


FIG. 1. (a) Active metabolic rate (AMR), (b) standard metabolic rate (SMR) and (c) aerobic metabolic scope (AS) of *Danio rerio* exposed to pyrolytic polycyclic aromatic hydrocarbons (PY PAH) in four treatments: control, 0-3X, 1X and 3X, where X is the environmental reference concentration of  $5.5 \mu\text{g PY PAH g}^{-1}$  of dry food for two different life stages: juveniles (□) and adults (■). Values are means  $\pm$  s.d.

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208           There was no significant difference in *SMR* among the four treatments. Even  
209 though the concentration of PAH and their metabolite compounds were not assessed in  
210 juveniles and adults in this study, previous work has shown that these concentrations  
211 tended to be proportional to the PAH content of the food received (Vignet *et al.*, 2014a).  
212 These results suggest that even the highest level of contamination tested (three times the  
213 average environmental concentration) was not sufficiently extreme to induce significant  
214 variation in *SMR*. Moreover, the lack of effect on *SMR* is contrary to the initial  
215 hypothesis, which stated that an increase in *SMR* was expected because of supplementary  
216 energy costs induced by PAH detoxification processes. Lannig *et al.* (2006) showed that  
217 a 40–86% increase of *SMR* can be induced in oysters *Crassostrea virginica* (Gmelin 1791)  
218 exposed to cadmium. This increase appears to be mostly due to the elevated costs of  
219 protein synthesis involved in detoxification or protective mechanisms such as cellular  
220 repair or expression of stress proteins.

221           In the previous study of Lucas *et al.* (2014a), *AMR* ranged between 0.92 and 0.94  
222 mg O<sub>2</sub>g<sup>-1</sup>h<sup>-1</sup> in juvenile and adult zebrafish. This is consistent with the current results for  
223 adults, while a slightly higher *AMR* was measured in juveniles (Fig. 1A). These results  
224 suggest that *D. Rerio* would not have a reduced capacity to sustain oxygen-demanding  
225 activities such as locomotion, digestion or growth (Claireaux & Lefrancois, 2007).

226           However, Vignet *et al.* (2014a) found a PAH dose-dependent reduction in growth  
227 despite the lack of effect of PAH on metabolism. This was probably mainly due to  
228 alteration of digestive capacity.

229           Despite numerous studies on fishes exposed to petroleum, there is no clear  
230 conclusion regarding effects on aerobic metabolism. In fact, some investigations on

231 persistent organic pollutants reported that fishes maintain their aerobic metabolism. The  
232 lack of effect in the present study is for instance in accordance with Milinkovitch *et al.*  
233 (2012) who observed no modification of *SMR*, *AMR* and *ASin* golden grey mullet *Liza*  
234 *aurata* (Risso, 1810) after exposure to crude oil and dispersants. Nor did McKenzie *et al.*  
235 (2007) find that organic pollutants affected metabolic rates of chub *Leuciscus*  
236 *cephalus*(Linnaeus, 1758). In contrast, other studies have demonstrated an increase (Hose  
237 & Puffer, 1984; Correa & Garcia, 1990; Davison *et al.*, 1992) or a decrease (Sharp *et al.*,  
238 1979; Serigstad & Adoff, 1985; Prasad, 1987; Davoodi & Claireaux, 2007; Christiansen  
239 *et al.*, 2010) of *ASin* fishes after petroleum exposure. However, fish contamination in  
240 these studies occurred by an aqueous pathway (involving the water soluble fraction of  
241 petroleum), which may count for the contrasting results with the present study. Such  
242 exposition may indeed have caused alterations to the gills epithelium leading to reduced  
243 oxygen diffusion into the blood (Claireaux *et al.*, 2004; Davoodi & Claireaux,  
244 2007). Therefore, the concentration and/or type of PAH tested in this present study did not  
245 induce impairments in the mechanisms involved in metabolic regulation.

246         However, it is also worth noting that organisms which suffer long-term chronic  
247 environmental stress can present physiological adaptations to maintain their homeostasis  
248 (Barton, 2002). Chronic exposure to PAH may have induced such adaptations in *D. rerio*.  
249 This study used the progeny of contaminated *D. rerio* (Lucas *et al.* 2014b). Even though  
250 no effects were observed on directly contaminated parents, an increase of *SMR* was  
251 observed in larval progeny of fish exposed to very high concentrations of PY PAH (the 3X  
252 treatment, Lucas *et al.*, 2014b). In addition, cardiac performance, heart rate and mRNA  
253 expression of genes encoding for cardiac activity were all modified at the environmentally

254 representative PAH concentration 1X (Lucas *et al.*, 2014b) Based on these results, the  
255 effects on larvae of parental exposure to PAHs worthy of further study, as parental  
256 exposure may impact aerobic metabolism as well as cardiac function (Lucas *et al.*,  
257 2014b). These results will improve the understanding of the potential effects of pyrolytic  
258 PAH on the physiology of *D. rerio* in particular, and fish in general.

259

## 260 **Acknowledgements**

261 The authors are grateful to Didier Leguay and Michel Prineau for their help  
262 during the experiment. All experiments were carried out at Ifremer (Plateforme  
263 d'Ecophysiologie des Poissons), La Rochelle station, France. This study was financially  
264 supported by the ANR project ConPhyPoP (CES 09\_002) and JL received a doctoral  
265 grant from the Regional Council of Poitou-Charentes. This study was conducted under  
266 the approval of the Animal Care Committee of France under the official licence to Marie-  
267 Laure Bégout (17-010).

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