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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 ± 0.5 °C, conductivity 300 ± 50 μScm^{-1} , air saturation
95 $\geq 80\%$, pH 7.5 ± 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), 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 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 $[\text{PAH}] = 5816 \pm 1433$ ngg^{-1}). Based on this reference, two other treatments were
110 tested: a lower concentration of $0.3X$ ($[\text{PAH}] = 1763 \pm 468$ ngg^{-1}) and a higher
111 concentration of $3X$ ($[\text{PAH}] = 18151 \pm 4983$ 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⁻¹ of tissue; 0.3X = 20 ngg⁻¹, 1X = 72 ngg⁻¹; 3X = 275
 118 ngg⁻¹; 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 μm , 315–500 μm , $\geq 500\mu\text{m}$). 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) 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⁻¹). 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 O₂g⁻¹h⁻¹, 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 O₂ l⁻¹) is the change in
175 oxygen concentration during the measurement period Δt (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).

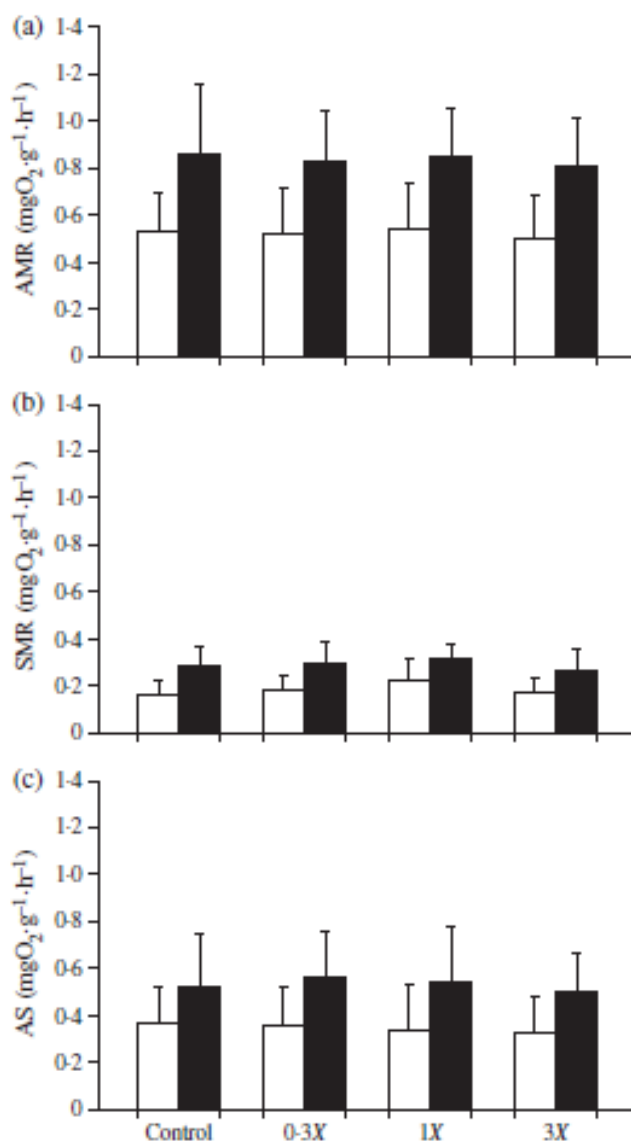


FIG. 1. (a) Active metabolic rate (AMR), (b) standard metabolic rate (SMR) and (c) aerobic metabolic scope (AS) of *Danio rerio* exposed to pyrolytic polycyclic aromatic hydrocarbons (PY PAH) in four treatments: control, 0-3X, 1X and 3X, where X is the environmental reference concentration of $5.5 \mu\text{g PY PAH g}^{-1}$ 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 O₂g⁻¹h⁻¹ 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

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268

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