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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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Abstract

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

Keywords: metabolic rates, static respirometry, sub-lethal concentration, petroleum hydrocarbons
Present as complex mixtures in environment, pyrolytic polycyclic aromatic hydrocarbons (PY PAH) result from combustion of organic matter and enter aquatic ecosystems through atmospheric deposition (Hylland, 2006). Due to their high liposolubility, PAH are typically adsorbed by organic matter or marine sediments, bioaccumulated by organisms at the lowest trophic levels (e.g. invertebrates; O’Connor & Lauenstein, 2006; Bustamante et al., 2012) and transferred through trophic chains (Hylland, 2006; Vignet et al., 2014a, 2014b).

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

This study aimed at investigating responses of fish exposed to PAH through the assessment of aerobic metabolic scope (AS) as an indicator of the physiological state of the organism (Fry, 1947). AS represents the amount of oxygen the animal is able to provide for all activities beyond standard metabolism (e.g. locomotion, digestion, feeding; Fry, 1947, 1971). It is defined as the difference between active metabolic rate (AMR), which is the highest metabolic rate the organism can sustain, usually during
maximal activity, and the standard metabolic rate (SMR), the metabolic rate necessary to maintain vital functions and measured under resting conditions at a known ambient temperature (e.g. Brett, 1964; Fry, 1947; White et al. 2006). AS is known to be modulated by pollutants (e.g. Sharp et al., 1979; Hose & Puffer, 1984; Correa & Garcia, 1990; Davison et al., 1992; Nikinmaa, 1992; Wilson et al., 1994; Lannig et al., 2006; Johansen & Jones, 2011; Davoodi & Claireaux, 2007; Christiansen et al., 2010). Focussing on PAH, Davison et al. (1992) reported that the Antarctic fish Pagophenia borchgrevinki (Boulenger, 1902) doubled its AS after exposure to an aqueous fraction of petroleum. Davoodi & Claireaux (2007) showed a 30% decrease of AS in the common sole Solea solea (Quensel, 1806) acutely exposed to a fuel. This AS reduction could be explained by malfunctions in organs involved in oxygen transport (e.g. heart, gills) and an associated decrease of AMR (Claireaux, 2004). Another possibility to reduce AS is the setting up of supplementary energy-demanding detoxification processes, which could increase SMR (Lannig, 2006).

This study aimed to determine the impacts of environmentally relevant concentrations of PY PAH on the aerobic metabolism of zebrafish Danio rerio (F. Hamilton, 1822) contaminated by ingestion. The main hypothesis was that chronic exposure to PY PAH would impair AS by increasing SMR and/or reducing AMR. The consequent potential reduction of AS would indicate a decrease in the capacity of the fish to support oxygen-demanding activities beyond SMR. Metabolic variables were assessed for two durations of chronic exposure, 2 and 6 months (juvenile and adult stages, respectively).
Pairsof *D. rerio* (wild-type Tuebingen strain) were reared together in 10 l tanks. Aquaria were filled with water prepared as a mixture of reverse osmosis-treated water and tap water, both filtered through sediment and activated charcoal filters. Rearing conditions were: temperature 28 ± 0.5 °C, conductivity 300 ± 50 µScm⁻¹, air saturation ≥ 80%, pH 7.5 ± 0.5, photoperiod of 14 h light/10 h dark. The fish were fed twice daily with commercial dry food (INICIO Plus, BioMar, www.biomar.com), occasionally supplemented with red sludge worms (Boschetto-Frozen fish food, www.achat-aquarium.fr). Over a period of one month, spawn was obtained weekly from pairs following the protocol described in Lucas *et al.* (2014a) and Vignet *et al.* (2014a). Spawn was then mixed to avoid any parental influence.

At two weeks old, fish were kept in groups of 30 individuals in 10 l aquaria. Contamination with PY PAH was achieved through the trophic pathway. Artificial dry food was contaminated with a mixture of PY PAH. The mixture was composed of 95% non-substituted PAH with a majority of four- and five-ring PAH; a detailed description is given in Vignet *et al.* (2014a). The PAH concentration targeted for contamination of pellets was 5000 ngg⁻¹, based on concentrations measured in molluscs in the Seine estuary. This reference environmental concentration is hereafter referred to as X; it represents one of the four treatments tested in this study (PAH concentrations measured in diet [PAH] = 5816 ± 1433 ngg⁻¹). Based on this reference, two other treatments were tested: a lower concentration of 0.3X ([PAH] = 1763 ± 468 ngg⁻¹) and a higher concentration of 3X ([PAH] = 18151 ± 4983 ngg⁻¹). A fourth (control) treatment was added in which dry food was exposed only to dichloromethane, the solvent used to carry the PAH. Contamination through food was achieved by feeding fish twice a day with one
of the four treatments. Quantification of hydroxylated metabolites in larvae at 15 days post fertilisation (dpf) indicated a dose-dependent increase of metabolites confirming successful contamination (total concentrations of hydroxylated metabolites for each treatment: control = 9.1 ng g⁻¹ of tissue; 0.3X = 20 ng g⁻¹, 1X = 72 ng g⁻¹; 3X = 275 ng g⁻¹; Vignet et al., 2014a). Fish were fed with treated pellets from their first meal (5 dpf) to the ages of 2 months for juveniles and 6 months for adults (Table 1) with size-adapted food (≤ 125µm, 125–315µm, 315–500µm, ≥ 500µm). In accordance with protocols in Vignet et al. (2014a), larvae were fed ad libitum and then, starting from two months old, the ration of food was 5% to 2% of the biomass in each tank in order to maintain constant growth. For all fish, brine shrimps (Ocean Nutrition Europe BVBA, http://www.oceannutrition.eu/fr/default.aspx) were given as supplementary food once a day. Characteristics of the fish are reported Table 1.

Table I. Biometry of juveniles and adults Danio rerio in each treatment (mean ± SE). X is the environmental reference concentration of 5.5 µg PY PAHs g⁻¹ of dry food. Control was food that had been exposed to dichloromethane only and did not contain PY PAHs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lifestage</th>
<th>Number of fish (n)</th>
<th>Weight (g)</th>
<th>Standard length (cm)</th>
<th>Total length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Juveniles</td>
<td>24</td>
<td>0.20±0.08</td>
<td>2.23±0.29</td>
<td>2.72±0.37</td>
</tr>
<tr>
<td></td>
<td>Adults</td>
<td>15</td>
<td>0.63±0.19</td>
<td>3.08±0.28</td>
<td>3.80±0.35</td>
</tr>
<tr>
<td>0.3X</td>
<td>Juveniles</td>
<td>23</td>
<td>0.21±0.12</td>
<td>2.18±0.27</td>
<td>2.73±0.20</td>
</tr>
<tr>
<td></td>
<td>Adults</td>
<td>12</td>
<td>0.68±0.11</td>
<td>3.26±0.20</td>
<td>3.94±0.20</td>
</tr>
<tr>
<td>1X</td>
<td>Juveniles</td>
<td>24</td>
<td>0.178±0.04</td>
<td>2.21±0.08</td>
<td>2.69±0.15</td>
</tr>
<tr>
<td></td>
<td>Adults</td>
<td>15</td>
<td>0.57±0.13</td>
<td>3.00±0.32</td>
<td>3.69±0.30</td>
</tr>
<tr>
<td>3X</td>
<td>Juveniles</td>
<td>24</td>
<td>0.18±0.08</td>
<td>2.18±0.29</td>
<td>2.65±0.33</td>
</tr>
<tr>
<td></td>
<td>Adults</td>
<td>15</td>
<td>0.44±0.16</td>
<td>2.84±0.40</td>
<td>3.36±0.41</td>
</tr>
</tbody>
</table>
To assess the aerobic metabolic rate of fish, eight identical circular size-adapted respirometers (diameter: 3.75 cm, volume: 0.061 l for juveniles and 7.50 cm, 0.179 l for adults) were employed. These were immersed in two buffer tanks (depth x length x height: 10 x 75 x 75 cm for both juveniles and adults) filled with temperature-controlled and aerated water. Oxygen consumption was measured by intermittent-flow respirometry (Steffensen, 1989) where the water supply in each respirometer was provided by flush pumps controlled by a timer. This system alternated phases of flushing and oxygen renewal with phases of measurement of oxygen consumption (MO$$_2$$), each of which lasted 30 min. Finally, a multichannel peristaltic pump was installed to create continuous water flow and ensure water mixing inside each of the chambers. Each respirometer was equipped with an optic fibre sensor (PreSens, www.presens.com) connected to a multichannel oxygen measuring system (OXY 4 mini, PreSens) to record the level of dissolved oxygen in the water. Oxygen data was sampled each five seconds with the program Oxyview (PreSens).

Fish were starved 24 h prior to respirometry. For each trial, eight fish (two in each treatment) were tested individually in respirometer, in two consecutive phases. First, to increase fish metabolism and assess AMR, each fish was transferred and chased with a stick in a 1 l tank (Schurmann & Steffensen, 1997; Lefrançois & Claireaux, 2003; Jourdan-Pineau et al., 2010; Clark et al., 2012; Cannas et al., 2013). When the fish was fatigued (did not respond to the stimulation), it was transferred into a respirometer. The oxygen consumption of the fish was immediately recorded for 30 min to calculate AMR. Then, to confirm the accuracy of the AMR assessment, each fish was chased again in the respirometer and its MO$_2$ measured again for a new period of 30 min.
The second step consisted of a resting period of 48 h to reach and estimate SMR. During this period fish were undisturbed and MO$_2$ was regularly and automatically measured. From these oxygen measurements, SMR was estimated according to the method described by Steffensen et al. (1994). Briefly, the frequency distribution of MO$_2$ values recorded during the last 24 h of the test was plotted. This generally produces a bimodal frequency distribution due to the routine activity of the fish. The higher mode (the first peak) is considered to reflect SMR and the lower mode (the second peak) corresponds to the routine metabolic rate (RMR), the energy required by the fish for maintenance plus random activity. During all the experiments, oxygen concentration was never lower than 75% oxygen saturation in each respirometer.

After the 48 h period of resting MO$_2$ measurements, fish were removed from the respirometers and anesthetised using benzocaine (50 mg l$^{-1}$). The length (standard and total length) and mass of each individual were determined (Table 1). To quantify microbial oxygen consumption in the respirometer, a blank measurement was carried out before and after each trial. A linear change in background MO$_2$ over the 48 h experimental trial was assumed and subtracted the expected value from the corresponding total MO$_2$ measured.

Oxygen consumption (MO$_2$), expressed in mg O$_2$ g$^{-1}$ h$^{-1}$, was calculated according to the formula $MO_2^{\text{meas}} = \Delta [O_2] V \Delta t \frac{M_{\text{meas}}}{l}$, where $\Delta [O_2]$ (in mg O$_2$ l$^{-1}$) is the change in oxygen concentration during the measurement period $\Delta t$ (in h), $V$ (in l) is the volume of the respirometer minus the volume of the fish, and $M_{\text{meas}}$ (in g) is the measured mass of the fish. An allometric relationship between oxygen consumption and body mass allows correction of MO$_2^{\text{meas}}$ using the formula $MO_2^{\text{cor}} = MO_2^{\text{meas}} (\frac{M_{\text{meas}}}{M_{\text{cor}}} - 1)^{\frac{1}{b}}$, where $MO_2^{\text{cor}}$ (in
mg O$_2$g$^{-1}$h$^{-1}$) is the oxygen consumption related to a standard fish of 1 g ($M_{cor}$), MO$_{2 meas}$
(in mg O$_2$g$^{-1}$h$^{-1}$) is the oxygen consumption estimated for experimented fish whose mass
was $M_{meas}$ (in g) and $b$ is the allometric scaling exponent describing the relationship
between oxygen consumption and body mass of fish. In previous study, $b$ was found to be
equal to 0.926 and 0.965 in the case of AMR and SMR assessment respectively (Lucas et al., 2014a)
Aerobic metabolic scope (AS) was calculated as the difference between AMR
and SMR. AMR, SMR and AS were assessed once for each individual.

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

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

This is the first study assessing the aerobic metabolism of zebrafish chronically
exposed to a mixture of pyrolytic PAH. Under these experimental conditions and at the
two life stages tested, trophic exposure to PY PAH did not affect SMR, AMR or AS of this
species. It is worth noting that these data for aerobic metabolism (Fig. 1B) are in
agreement with previous studies carried out for the same species and with a similar experimental approach (SMR = 0.19 mg O$_2$g$^{-1}$h$^{-1}$ in Barrionuevo & Burggren, 1999; SMR = 0.31 ± 0.11 mg O$_2$g$^{-1}$h$^{-1}$ and 0.35 ± 0.16 mg O$_2$g$^{-1}$h$^{-1}$ in juveniles and adults, respectively, in Lucas et al., 2014a).
There was no significant difference in SMR among the four treatments. Even though the concentration of PAH and their metabolite compounds were not assessed in juveniles and adults in this study, previous work has shown that these concentrations tended to be proportional to the PAH content of the food received (Vignet et al., 2014a). These results suggest that even the highest level of contamination tested (three times the average environmental concentration) was not sufficiently extreme to induce significant variation in SMR. Moreover, the lack of effect on SMR is contrary to the initial hypothesis, which stated that an increase in SMR was expected because of supplementary energy costs induced by PAH detoxification processes. Lannig et al. (2006) showed that a 40–86% increase of SMR can be induced in oysters Crassostrea virginica (Gmelin 1791) exposed to cadmium. This increase appears to be mostly due to the elevated costs of protein synthesis involved in detoxification or protective mechanism such as cellular repair or expression of stress proteins.

In the previous study of Lucas et al. (2014a), AMR ranged between 0.92 and 0.94 mg O$_2$g$^{-1}$h$^{-1}$ in juvenile and adult zebrafish. This is consistent with the current results for adults, while a slightly higher AMR was measured in juveniles (Fig. 1A). These results suggest that D. Rerio would not have a reduced capacity to sustain oxygen-demanding activities such as locomotion, digestion or growth (Claireaux & Lefrancois, 2007).

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

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

However, it is also worth noting that organisms which suffer long-term chronic environmental stress can present physiological adaptations to maintain their homeostasis (Barton, 2002). Chronic exposure to PAH may have induced such adaptations in D. rerio. This study used the progeny of contaminated D. rerio (Lucas et al., 2014b). Even though no effects were observed on directly contaminated parents, an increase of SMR was observed in larval progeny of fish exposed to very high concentrations of PY PAH (the 3X treatment, Lucas et al., 2014b). In addition, cardiac performance, heart rate and mRNA expression of genes encoding for cardiac activity were all modified at the environmentally
representative PAH concentration 1X (Lucas et al., 2014b) Based on these results, the effects on larvae of parental exposure to PAHs is worthy of further study, as parental exposure may impact aerobic metabolism as well as cardiac function (Lucas et al., 2014b). These results will improve the understanding of the potential effects of pyrolytic PAH on the physiology of D. rerio in particular, and fish in general.

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descendants of zebrafish exposed to pyrolytic polycyclic aromatic hydrocarbons. Environmental Science and Pollution Research 21, 13888–13897.


