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

Lucas Julie [1, 2, *] , Perrichon Prescilla [1, 2], Nouhaud Marine [1], Audras Alexandre [1], Leguen Isabelle [3], Lefrancois Christel [1]

[1] Inst Littoral & Environm, UMR Littoral Environm Soc LIENSs 7266, F-17000 La Rochelle, France.
[2] IFREMER, F-17137 Lhoumeau, France.
[3] INRA, LPGP Fish Physiol & Genom UR1037, F-35000 Rennes, France.

* Corresponding author : Julie Lucas, email address : julie.lucas@univ-lr.fr

Abstract :

The increase of anthropogenic activities on coastal areas induces discharges of polycyclic aromatic hydrocarbons (PAHs) in aquatic ecosystem. PAH effects depend not only on their concentration and the way of contamination but also on the different developmental stages of the organism. Zebrafish were exposed to relevant concentration of pyrolytic PAHs from the first meal (i.e., 5-day post fertilization, dpf) to mature adults. Parental effect of this type of exposure was evaluated through the assessment of aerobic metabolic scope, cardiac frequency, and cardiac mRNA expression on larval and/or embryo progeny of contaminated fish. Our results suggest that cardiac frequency increased in larval descendants of fish exposed to the environmental concentration of pyrolytic PAHs (i.e., 5 ng.g(-1) of food), while a lack of effect on aerobic metabolism in 5 dpf larvae was highlighted. A surexpression of mRNA related to the cardiac calcium transporting ATPase atp2a2a, a protein essential for contraction, is in accordance with this increasing cardiac frequency. Even if cardiac development genes cmlc1 and tnnt2a were not affected at early life stages tested, complementary work on cardiac structure could be interesting to better understand PAHs action.

Keywords : Parental transfer, Pyrolytic hydrocarbons, Sub-lethal concentration, Metabolic rate, Heart

Introduction

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

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

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

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

Material & Methods

Protocol of food contamination

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

Zebrafish exposure

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

Metabolites quantification

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

Table 1 Metabolites concentrations in larvae F_1 from genitors contaminated with pyrolytic PAHs. Mean ± SD in $ng.g^{-1}$ of larvae, n.d. not detected.

Metabolites	*Control*	0.3*X*	1*X*	3*X*
ΣHydroxynaphtalene (1+2 OHNaph)	nd	nd	nd	nd
2 Hydroxybiphenyle (2OHBi)	<blank	<blank	12	16.1
ΣHydroxyphenanthrene (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
ΣHydroxybenzo(a)pyrene (3+9 OHBaP)	nd	nd	9.1	34.6
Sum Hydroxy-PAHs	2	7.3	39	136

Respirometry

Experimental set-up

The set-up consisted of 8 independent glass micro respirometer chambers (diameter *d*= 1.12 cm, volume *V*= $0.98.10^{-3}$ l; Loligo systems, Denmark). Respirometers were submerged into buffer tanks (depth x length x height: 10x20x31 cm) and filled with oxygenated E3 solution at a constant temperature of 28°C. Peristaltic pumps controlled the water supply from the buffer tank to each respirometer. Each pump was controlled by a timer, allowing intermittent flow respirometry (Steffensen 1989), where phases of oxygen renewal alternated with phases of oxygen consumption measurements with a period of 15:15 min. Each respirometer was equipped with a fiber optic sensor (PreSens) connected to a multichannel oxygen measuring system (OXY 4 mini, PreSens) to record dissolved oxygen levels. Optic fibers were calibrated at 0% and 100% of air saturation at a temperature of 28°C. A factor of conversion based on oxygen solubility into water was used to convert oxygen data from percentage saturation to $mgO_2.l^{-1}$ (i.e. 100% was equivalent to 7.94 $mgO_2.l^{-1}$ for a 28°C temperature and a 0 salinity). Oxygen saturation was recorded every five seconds with the program Oxyview (PreSens).

Experimental protocol

Each experimental trial consisted in testing simultaneously 4 groups of 10 larvae F_1 whose genitors were exposed to one of the four treatments (*0.3X*, *1X*, *3X* and *control*). A total of 36 groups was tested ($n_{control}$=9 groups, $n_{0.3X}$=8 groups, n_{1X}=12 groups, n_{3X}=7 groups). Each experimental trial involved two steps. First, to assess active metabolic rate (*AMR*), metabolism of larvae F_1 was increased through chasing (Schurmann and Steffensen 1997; Lefrançois and Claireaux 2003; Jourdan-Pineau et al. 2010; Cannas et al. 2013, Clark et al. 2012). The tested groups of 10 larvae were chased with a stick into petri dishes. When exhausted (i.e. when fish did not respond to stimulation), each group was transferred into one of the 4 respirometers to assess *AMR* through a first oxygen consumption over the next 15 min. Second, to estimate standard metabolic rate (*SMR*), larvae were left undisturbed in the respirometer for 48h, during which oxygen consumption was regularly and automatically measured. After this period of measurements, larvae were removed from the respirometers and slightly

anesthetized with benzocaïne at a concentration of 50mg.l^{-1}. The body mass of each individual was determined using a microbalance. The tested groups showed homogenous weights; the average 'group weight' being *M*= 0.20± 0.02 mg (mean ± S.E). Before and after each trial, a blank measurement was performed to quantify microbial oxygen consumption in the respirometer. The average of these 2 values was substracted from the measured oxygen consumption. Each group of larvae was tested once.

Calculations

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

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

where $\Delta[O_2]$ (in $mgO_2.l^{-1}$) is the variation in oxygen concentration during the measurement period Δt (in h) and V (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 the corresponding tested group of 10 larvae .

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

$$MO_{2cor}=MO_{2meas}.(M_{meas}.M_{cor}^{-1})^{1-b} \quad \textit{equation (2)}$$

where MO_{2cor} (in $mgO_2.g^{-1}.h^{-1}$) is the oxygen consumption related to a standard fish of 1g (M_{cor}), MO_{2meas} (in $mgO_2.g^{-1}.h^{-1}$) 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. We used b_{AMR}=0.926 for the correction of active metabolic rate and and b_{SMR}=0.965 for the correction of standard metabolic rate (Lucas et al., 2014).

AMR was assessed as the maximal MO_2 obtained after chasing the larvae. *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 hours of the test was plotted. This generally produces a bimodal frequency distribution due to the routine activity of the fish. The higher mode (*i.e.* the first peak, Fig. 1) is considered to reflect the *SMR* and the lower mode (*i.e.* the second peak, Fig. 1) corresponds to the routine metabolic rate (*RMR*), *i.e.* the energy required by the fish for spontaneous activity. Aerobic metabolic scope AMS was calculated as the difference between *AMR* and *SMR*. *AMR*, *SMR* and AMS were assessed for each group.

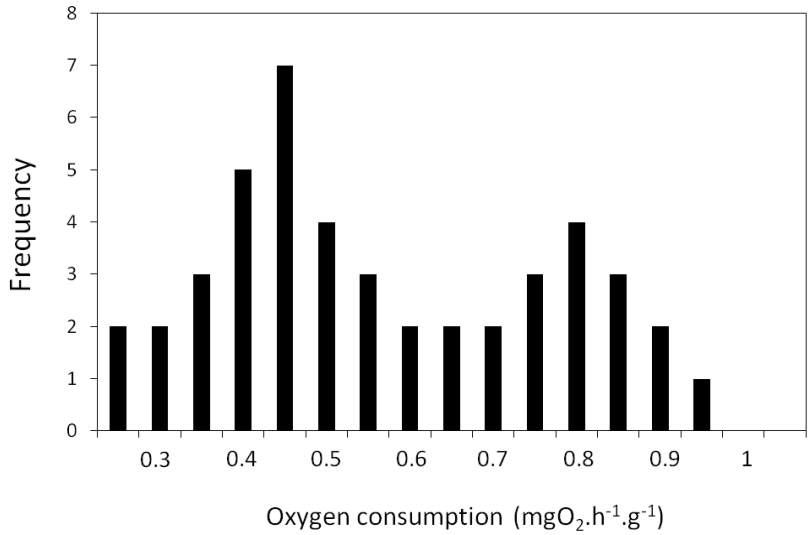


Fig. 1 An example of frequency distribution of oxygen consumption in larvae. The left and right peaks represent standard metabolic rate SMR and routine metabolic rate RMR, respectively

Cardiac frequency

Experimental protocol

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

Videos analysis

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

Cardiac mRNA expression

Transcript abundance of some genes encoding key proteins in cardiac development and activity (cardiac myosin light chain 1 (*cmlc1*), cardiac troponin type T2A (*tnnt2a*) and calcium transporting ATPase (*atp2a2a*) was examined in embryo (48hpf) whose genitors were exposed to 1*X* and control treatments and larvae F_1 (6dpf) from genitors exposed to 0.3*X*, 1*X* and control treatments.

Preparation of total RNA

Fish were euthanized and frozen on liquid nitrogen prior to storage at -80°C. Total RNA from pools of 30 embryo (48hpf) or 15 larvae F_1 (6dpf) was extracted using TRIzol (Invitrogen Ltd, Cergy Pontoise, France). Five to six pools were used per treatment (0.3*X*, 1*X* and *control*). Due to a limited number of embryos from genitors exposed to 3*X* treatment, it was not possible to measure mRNA expression in these descendants. RNA was quantified by measuring the optical density at 260 nm (OD_{260}). RNA integrity was checked using the Bioanalyzer 2100 Agilent and RNA 6000 LabChip® kits (Agilent Technologies, Waldbroon, Germany) according to the manufacturer's instructions

Real time RT-PCR

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

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

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

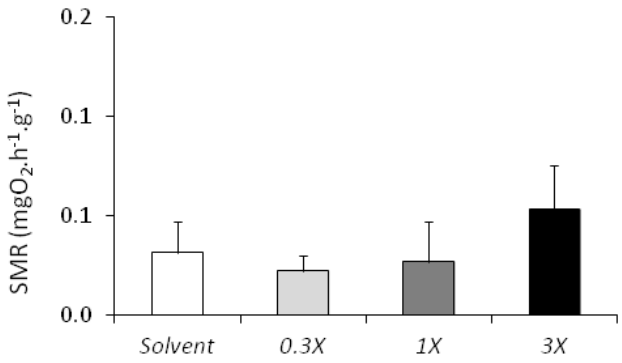
Statistical analysis

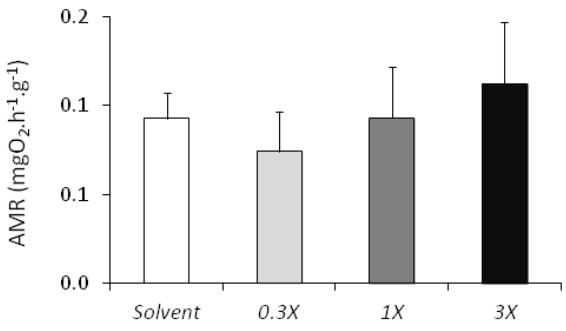
The statistical analysis was carried out using Statistica software. For analyses regarding aerobic metabolism and cardiac frequency variables, the normality and homoscedaticity were checked through the Shapiro and Barlett tests, respectively. These conditions, necessary to apply parametric tests, were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

Results

Active metabolic rate, Standard metabolic rate, Aerobic metabolic scope

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





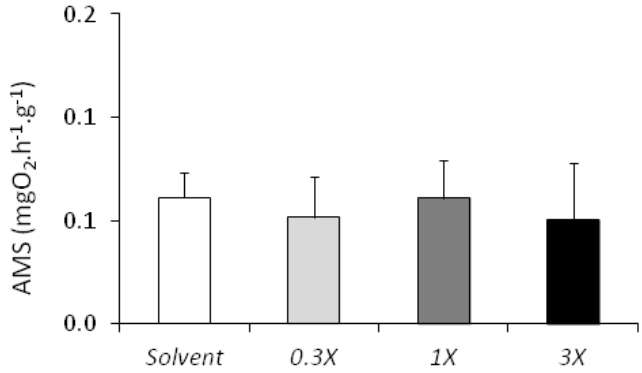


Fig. 2 Active metabolic rate (AMR, A), standard metabolic rate (SMR, B), and aerobic metabolic scope (AMS, C) of 5 dpf larvae F1 zebrafish *Danio rerio* coming from genitors exposed to one of the following treatments: control, 0.3X, 1X, and 3X. The environmental reference concentration X was 5.5 µg.g−1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

Cardiac frequency

PAHs exposure induced a significant effect on the cardiac frequency f_H measured in the 5dpf larvae F_1 (Fig 3; ANOVA, ddl=3, F=32.61, p<0.01). Post hoc test revealed that only cardiac frequency of larvae born from 1*X* genitors differed significantly from *control* larvae with in average f_H = 183.8±9.7 bt.min^{-1} (Tuckey test, p<0.01).

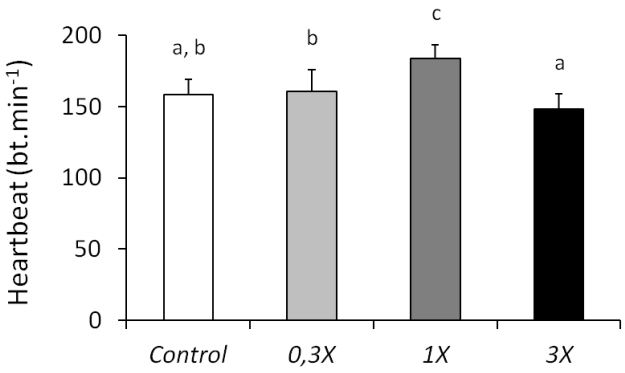


Fig. 3 Cardiac frequency of 5 dpf larvae F1 zebrafish *Danio rerio* coming from genitors exposed to one of the following treatments: control, 0.3X, 1X, and 3X. The environmental reference concentration X was 5.5 µg.g−1 of dry food. Mean values±standard deviation are plotted and treatments with common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

Cardiac mRNA expression

The mRNA abundance of cardiac muscle genes measured in 48hpf embryo F_1 was similar between embryo from genitors exposed to *1X* pyrolytic PAHs or control (Fig. 4). In 6dpf larvae F_1, mRNA expression for myosin light chain 1 and troponin type T2A was similar in larvae F_1 whatever the diet the genitors were exposed to (Fig 5). The mRNA expression of calcium transporting ATPase atp2a2a gene showed a different pattern. This gene was indeed upregulated in larvae F_1 whose genitors were exposed to *1X* pyrolitic PAHs (Mann-Whithney U test, p<0.01).

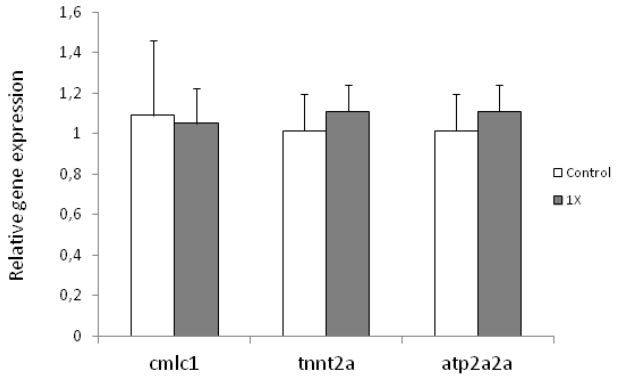


Fig. 4 Relative mRNA expression of three cardiac structural genes in 48 hpf embryo F1 zebrafish Danio rerio from genitors exposed to pyrolytic PAHs 1X treatment. Mean values ±standard deviation are plotted (N=6 pools of 30 embryo for control and N=5 for 1X treatment)

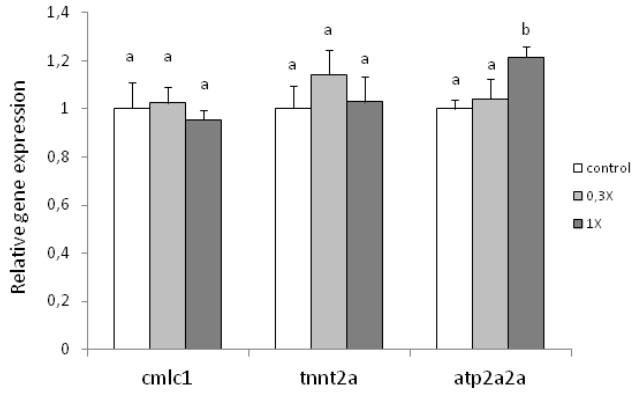


Fig. 5 Relative mRNA expression of three cardiac structural genes in 6 dpf larvae F1 zebrafish *Danio rerio* from genitors exposed to pyrolytic PAHs 0.3X and 1X treatment. The environmental reference concentration X was 5.5 µg.g−1 of dry food. Mean values±standard deviation are plotted (N=5 pools of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

Discussion

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

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

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

On the contrary, complementary work showed a similar mRNA expression of cardiac structural gene (cardiac myosin light chain 1 (*cmlc1*) and cardiac troponin type T2A (*tnnt2a*) in the progeny of parents exposed or not to PAHs treatments. This suggests a correct cardiac morphogenesis as it was observed on larvae used for cardiac frequency measurements. The pattern observed in the present study differs from recent studies investigated cardiotoxicity of PAHs in early life-stages of zebrafish directly exposed to PAHs by sediment contacts or oil. Cardiac defects observed were indeed principally heart deformities (e.g. pericardial oedema), arrhythmia or bradycardia, and never tachycardia (Incardona et al. 2003, 2004, 2005, 2006, 2011; Hicken et al. 2011; Perrichon, personal communication). However, comparison is limited since (i) most of these investigations have tested individual compounds at unrealistic environmental concentrations in order to trigger toxic mechanisms that impair physiological or metabolic functions (Incardona et al. 2005, 2006, 2011) and (ii) fish were directly exposed to pollutants whereas in the present study contamination was expected to occur by parental transfer. Moreover, depending on the way of exposure, PAHs levels of contamination in organisms are expected to be different. In the present study, PAHs-metabolites concentrations measured in larvae F_1 tended to be proportional to the PAHs degree of exposure but the levels of contamination are low. Focusing on fish directly exposed, PAHs-metabolites have not been assessed in genitors at 4 months but Vignet et al., (in press) measured it on 15 dpf larvae. The concentrations tended to be proportional to the PAHs content of the food they received 9.1±0.62, 19.3±2,91, 72±11,79, 275.11±50,14 for *control*, 0.3*X*, 1*X*, 3*X* respectively. These differences in PAHs-metabolites concentrations may be explained by the fish capacity to quickly metabolize PAHs in more water-soluble components easier to excrete (Blahova *et al.*, 2013, Dévier *et al*., 2013; Tuvikene, 1995). Probably only a small part of these hydrocarbons derivates has been accumulated in genitor's gonads and transferred into the eggs of zebrafish, as shown in several studies in aquatic animals (Lotufo, 1998; Miller, 1993; Monteverdi and Giulio, 2000; Hall and Oris, 1991). The lack of information regarding PAHs parental transfer in literature and these PAHs-metabolites concentrations may participate to the fact that no clear pattern regarding the effect of parental exposure to PAHs on the progeny cardiac frequency can be established.

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

(Perrichon, personal communication).

Aerobic metabolism of the progeny coming from genitors directly exposed to PAHs never differed significantly compared to control; even for the treatment *1X* for which cardiac frequency was significantly influenced. Very few studies have examined influence of PAHs exposure on cardio-respiratory performance (e.g. Claireaux and Davoodi, 2010, Hicken 2011). Associated to a decrease in cardiac performance, Claireaux and Davoodi (2010) have observed that fuel-exposed common sole displayed lower aerobic metabolism compared to control. Hicken et al. (2011) also demonstrated that early exposure to crude oil altered cardiac morphology (by modifying ventricular shape), which reduced cardio-respiratory performance and aerobic capacity in adult zebrafish. On the contrary, in the present study, variations in aerobic metabolism and cardiac frequency did not match in the larvae tested (Fig. 2 and 3). This may have different explanations. First, it is worth noticing that cardiac frequency is only one cardiac parameter on which depends the oxygen transport. Taking into consideration other parameters, such as the stroke volume, should give a better overview of an organism cardiac performances. Second, the proportion of cutaneous respiration is expected to be high in early life stage, which can distort the oxygen transport capacity. Third, larvae were tested in group of 10 individuals, while cardiac frequency was measured in individuals. All these reasons may also count for the different results observed later in 2-months juveniles from the same generation F_1 for which it was observed a significant decrease in both *AMR* and AMS in the treatment *3X* (Lucas et al., *submitted*). These juveniles were indeed individually tested, which allowed more acute measurements of metabolic rates.

Conclusion

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

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Figures captions

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

Figure 2. Active metabolic rate (*AMR*, *A*), standard metabolic rate (*SMR*, *B*) and aerobic metabolic scope (*AMS*, *C*) of 5dpf larvae F_1 zebrafish *Danio rerio* coming from genitors exposed to one of the following treatments: 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), treatment with common superscript are not significantly different.

Figure 3. Cardiac frequency of of 5dpf larvae F_1 zebrafish *Danio rerio* coming from genitors exposed to one of the following treatments: control, 0.3*X*, 1*X* and 3*X*. **The environmental reference concentration *X* was 5.5 µg.g^{-1} of dry food.** Mean values ±standard deviation are plotted and treatments with common superscript are not significantly different.

Figure 4. Relative mRNA expression of three cardiac structural genes in 48hpf embryo F_1 zebrafish *Danio rerio* from genitors exposed to pyrolytic PAHs *1X* treatment. Mean values ± standard deviation are plotted (N=6 pools of 30 embryo for control and N=5 for 1*X* treatment).

Figure 5. Relative mRNA expression of three cardiac structural genes in 6dpf larvae F_1 zebrafish *Danio rerio* from genitors exposed to pyrolytic PAHs 0.3*X* and 1*X* treatment. **The environmental reference concentration *X* was 5.5 µg.g^{-1} of dry food.** Mean values ± standard deviation are plotted (N=5 pools of 15 larvae for each treatment). For each gene, treatments with common superscript are not significantly different.