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Environmental Science and Pollution Research
December 2014, Volume 21(24), Pages 13877-13887
http://dx.doi.org/10.1007/s11356-014-2585-5
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The original publication is available at http://www.springerlink.com

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Long-term disruption of growth, reproduction, and behavior after embryonic exposure of zebrafish to PAH-spiked sediment

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Abstract:

A natural sediment spiked with three individual polycyclic aromatic hydrocarbons (PAHs; pyrene, phenanthrene and benzo[*a*]pyrene) was used to expose zebrafish embryos and larvae during 4 days. The total PAH concentration was 4.4 μg g^{-1} which is in the range found in sediment from contaminated areas. Quantification of metabolites in the larvae after exposure confirmed the actual contamination of the larvae and indicated an active metabolism especially for pyrene and benzo[*a*]pyrene. After a transfer in a clean medium, the larvae were reared to adulthood and evaluated for survival, growth, reproduction, and behavior. Measured endpoints revealed a late disruption of growth (appearing at 5 months) and a trend toward a lower reproductive ability. Adults of embryos exposed to sediment spiked with PAHs displayed lethargic and/or anxiety-like behaviors. This latter behavior was also identified in offspring at larval stage. All together, these effects could have detrimental consequences on fish performances and contribution to recruitment.

Keywords: Danio rerio ; Polycyclic aromatic hydrocarbon ; Lifecycle ; Anxiety-like behavior ; Offspring

1. Introduction

Among organic pollutants, polycyclic aromatic hydrocarbons (PAHs) constitute a diverse family of molecules made of the association of at least two aromatic rings. PAHs emission in the environment is directly linked to human activity and increased over the last decades (Shen et al. 2013). PAHs are produced during the incomplete combustion of organic matter and are naturally abundant in oils. PAHs are always present in the environment as complex mixtures, the composition of which depends on contribution from pyrolytic or petrogenic sources. Pyrolytic mixtures are characterized by higher levels of heavy PAHs such as benzo[a]pyrene (BaP) while petrogenic mixtures contain lesser amounts of heavy PAHs and greater amounts of light PAHs and alkylated-PAHs (Latimer & Zheng 2003). Pyrolytic PAHs are emitted in the atmosphere and deposited in water or soils (Shen et al. 2013). Petrogenic PAHs can be directly discharged in water in case of oil spill or as a result of naval or offshore oil drilling activities or natural oil leaks. They can also be released on soils as a consequence of human activities. Soil deposited PAHs can enter water compartment after runoff (Hylland 2006, Latimer & Zheng 2003). Because they are hydrophobic molecules, PAHs in water are associated with suspended particulate matter to end into sediments (Hylland 2006). Monitoring networks have documented PAHs concentrations of up to 50 µg g^{-1} dry weight (dw) in sediment from various affected aquatic ecosystems even if concentration of highly contaminated areas is rather in the 10 µg.g^{-1} range (Baumard et al. 1998, Benlahcen et al. 1997, Cachot et al. 2006, Johnson et al. 2007, Varanasi et al. 1993, Yanagida et al. 2012). Sediment therefore constitutes a major sink for hydrophobic pollutants and can act as secondary sources for aquatic systems contamination (Hylland 2006).

In both freshwater and marine ecosystems, a number of fish lay their eggs on sediments or gravel in river or seabed, including smelt, salmonids, capelin and some minnows. Some others, such as killifish, even bury their eggs in sediment. Analytical assessment of PAHs transfer through the chorion has been demonstrated when embryos are exposed directly through water (Hornung et al. 2007) or to a spiked sediment (Cachot et al. 2007, Djomo et al. 1996, McElroy et al. 2006). There are therefore high probabilities that embryos developing on PAHs contaminated sediment become contaminated and hence affected during embryonic development when several ontogenetic processes are occurring. Such fish early life stages (ELS) are very sensitive to modification of their environment. A large number of organic xenobiotics *e.g.* dioxins, PCBs, PBDEs have been experimentally reported to disrupt embryonic development (Carney et al. 2006, Costa & Giordano 2007, Hornung et al. 1996, McClain et al. 2012, Usenko et al. 2011, Xiong et al. 2008). This is also the case for aqueous exposure to individual PAHs or oil mixtures (Carls et al. 2008, He et al. 2012, Hendon et al. 2008, Incardona et al. 2009, Incardona et al. 2005, Incardona et al. 2004, Incardona et al. 2006, Shi et al. 2012), through contact with a spiked- or natural sediment (Cachot et al. 2007, Debruyn et al. 2007, Le Bihanic et al. *Submitted-b,* Le Bihanic et al. *Submitted-c,* Yang et al. 2010) or sediment affected by oil spills (Debruyn et al. 2007, Frantzen et al. 2012, Hicken et al. 2011, Hose et al. 1996, Incardona et al. 2009).

Numerous studies have described effects of PAHs on development of embryonic or larval stages, including oedema, altered development of the heart and the jaw (Carls et al. 1999, Debruyn et al. 2007, Heintz et al. 1999, Incardona et al. 2004, Li et al. 2011, Marty et al. 1997, Shi et al. 2012). However, very few publications have reported consequences of exposures of ELS of fishes to PAHs on later stages of development. Indeed only a prolonged (or delayed) reduction of growth (Cachot et al. 2007, Carls et al. 1999, Heintz et al. 1999) and reduction of aerobic capacity in adults (Hicken et al. 2011) after exposure of the ELS stage to PAHs have been reported.

In order to evaluate the late consequences of an early and short exposure to PAHs on fish physiological variables, we have exposed zebrafish ELS to a spiked sediment with a mixture

of 3 PAHs used at environmental concentrations : Pyrene (Pyr), phenanthrene (Phe) and benzo[a]pyrene (BaP) are abundant PAHs in contaminated river sediments where they represent 20 to 27% of total quantified PAHs (Cachot et al. 2006). They are also representative of PAHs variety as of ring number, from 3 to 5. After exposure, larvae were transferred to a clean medium and reared until reproduction. Growth was monitored as well as reproduction and behavior in adults. Behavior of offspring was also monitored at larval stage.

2. Materials and methods

This study was conducted under the approval of the Animal Care Committee of France, under the official licence of M.-L. Bégout (17-010).

2.1. Sediment spiking and chemical characterisation

The reference sediment was collected in March 2010 at Yville-sur-Seine (Seine-Maritime, France) in a former gravel pit. This site was chosen because sediment was previously shown to be marginally contaminated by organic and metallic pollutants (Cachot et al. 2006) and exposure to this sediment did not produce measurable toxic effects on fish embryos or at latter stage (Cachot et al., 2007). The sediment was freeze-dried and then ground in a mortar to obtain a thin homogeneous particulate matter as described in (Vicquelin et al. 2011). The freeze-dried reference sediment was spiked with Pyr, Phe and BaP solutions (Sigma-Aldrich, France) in dichloromethane (solvent) in order to obtain theoretical concentrations of 2750 ng.g^{-1} dw of sediment for Phe, 2350 ng.g^{-1} dw for Pyr and 1250 ng.g^{-1} dw for BaP. Sediment spiking and subsequent characterisation were performed as described in (Vicquelin et al. 2011). Control sediment was prepared from the same freeze-dried sediment treated as indicated above, including solvent but excluding PAHs solutions.

2.2. Fish strains, rearing, embryo production and exposure

We used one common laboratory strain: the AB strain (ZFIN ID: ZDB-GENO-960809-7) derived from individuals crossed in 1970 in Eugene (Oregon, USA). This strain has been established in our laboratory for 6 years as large batches of individuals originating from Amagen platform (Gif/Yvette, France) and Pasteur Institute fish facility (Paris, France). Adults were maintained at 27°C in a controlled 14 hour light/10 hour dark (14:10) photoperiod in the same rack and were fed *ad libitum* twice a day with pellets (INICIO Plus 0.5, Biomar, France) between 9:00 and 9:30 in the morning and 16:30 and 17:30 in the afternoon and once with artemias (INVE) between 11:30 and 12:30. Eggs were obtained by random pairwise mating of zebrafish. One adult male and one female were placed together in spawning boxes the evening before eggs were required (AquaSchwarz, Germany). Eggs were collected in the morning and the fertilisation rate assessed within two hours of collection: only spawns with a fertilisation rate above 80% were kept. At the same time, spawns were sorted to remove faeces, and dead or unfertilised embryos. A minimum of 5 spawns were mixed and 30 eggs were distributed for each replicate on a Nytex thermoformed basket at 4 hours post fertilization (hpf) and inserted in a 3 cm diameter Petri dish containing 3 g dw of sediment and 4 ml of E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4).

Exposures were repeated as independent triplicates hence providing each time 3 replicate tanks (hereafter called ‘replicate of origin’ in the data analysis). A total of 17 triplicates were produced over a three months period, for both Control and Exposed treatments, using 110 spawns issued from 250 spawning pairs. All these replicates were used to monitor survival, lethal and sublethal endpoints as listed in Table 1 in (Lammer et al. 2009). Out of the 17

triplicates; one triplicate of each condition (Control and Exposed) was used for metabolites quantification and two triplicates of each condition kept for rearing and further analyses. Embryos and larvae were further maintained at 28°C in Petri dishes in an incubator with the same light/dark cycle as adults. After hatching, chorions were removed manually. Exposure was continued until 96 hpf, thereafter; larvae were transferred in clean E3 medium in a new Petri dish. At 5 (days post fertilization) dpf, larvae were transferred from Petri dish to 1L tanks (spawning box without inserts; AquaSchwarz, Germany) containing 100 mL of E3 solution. System water was then progressively added (150 ml each day) until tanks were full. At 30 dpf larvae were then transferred in a dedicated rack and reared until the adult stage. Larvae were fed with plain artemias (INVE) and dry food (grinded to appropriate size from INICIO Plus 0.5 mm, Biomar, France) from 5 days post fertilisation (dpf) onwards. This generation is hereafter called F0.

2.3. Metabolites quantification after exposure

At the end of exposure, 30 larvae were euthanized with a lethal dose of benzocaine (250 $mg.l^{-1}$) collected in microtubes. A maximum of liquid was removed and tubes frozen in liquid nitrogen. Larvae were grinded in acetate buffer at pH 5 and metabolites were quantified in whole larvae according to published protocol (Devier et al. 2013, Le Du-Lacoste et al. 2013).

2.4. Survival and growth of F0 fish

Larvae and fish death was monitored daily. Fish growth was monitored monthly starting at 3 months post fertilisation (mpf, age factor in the downstream analysis) onward. Fish were anaesthetized for 1 min in benzocaine (50 $mg.l^{-1}$) and individual standard length (from the tip of the head to the beginning of caudal fin rays; cm, to the nearest mm) and body mass (g, to the nearest mg) were measured. Fish were sexed when possible (on the basis of morphological clues).

2.5. Reproduction of F0 fish

Reproduction was monitored 2-3 times a week during 5 weeks starting at 3 mpf. Spawns were obtained by placing a spawning box (AquaSchwartz, Göttingen, Germany) within each tank starting at 3 mpf in the late afternoon. Spawning boxes were inspected the next morning and eggs were collected. Number of fertilized eggs were counted and normalized by the number of female in each tank and by the number of time spawning boxes were introduced into the tank (hereafter named spawning request). Survival of F1 offspring larvae was monitored over 10 days.

2.6. Behavioural experiments

Behavioural experiments were performed using 6 month-old adult F0 males and 5 dpf F1 larvae (offspring), in a dedicated room kept at 27°C ± 1°C, with a 14:10 photoperiod synchronised with the rearing room so as to minimize unwanted correlated effects. Daylight started at 08:30 and there were no twilight transition periods. At the end of each recording session, adult fish were anaesthetized for 1 min in benzocaine (50 $mg.l^{-1}$) and individual standard length (cm) and body mass (g) were measured.

2.7. 24-h swimming activity and photomotor response in F0 adults.

Fish from each treatment were randomly placed at each session in 3-L tanks (24.5 × 15 × 13.5 cm, AquaBox® 3, AquaSchwartz, Göttingen, Germany) filled with 1.5 L of system water, to avoid tank position and session bias. The 12 tanks were isolated from neighboring tanks by opaque walls. The set-up was placed on top of an infrared apparatus (IR floor 1 × 1 m, Noldus, The Netherlands). During the day, the room was lit with two halogen spotlights (Philips 80 W, illumination of 30 lx. near the tank). During the night, the spotlights were turned off and infrared light from the floor was used to record fish movements (illumination <1 lx. near the tank). Fish were placed in their tanks at 17:00 the day before the experiment, for one night of acclimatization. Recording started the next day at 12:30 and lasted 24 h. For one session, the camera was placed above the tanks to monitor horizontal movements. For the other session, the camera was placed in front of the tanks to record vertical movements. For this side view; the tanks were placed on a four-level shelf in front of the vertically placed IR floor. Swimming path length (top view) and time spent in each third of the tank section (side view) were measured. We challenged 12 control fish and 8 exposed fish from each of the three experimental replicate and different fish were used for top and side monitoring. The water was changed after each session.

In addition to the 24-h swimming activity measurement, these experiments were designed to monitor photomotor responses used as an indicator of complex phenotypes of stress and anxiety. At the end of the 24-h recording, fish activity was further recorded for 1 h (Light on-1), then they were challenged with a sudden dark period (15 min, Light off) and video recordings continued for an additional period of 1h30 (Light on-2). Swimming path length (top view) and time spent in each third of the tank section (side view) were measured. For both protocols, 36 control and 24 exposed fish were recorded.

2.8. Photomotor response in F1 5 dpf larvae

At 17:00 the day before the challenge (4 dpf), single larvae were transferred to the cells of a 24-well plate (Krystal 24, opaque wall and clear bottom micro-plate) where they were arranged in a mixed design and visually isolated from each other. Equal number of larvae from each treatment was introduced in each plate to avoid any trial effect. The 24-well plates were kept overnight in the breeding incubator. The following day, two hours before the challenge, well plate was transferred to the video acquisition room and placed on top of a size-matched infrared floor, which allowed the larvae to be filmed under both light and dark conditions (Vignet et al. 2013). The 15-min challenge included three 5-min periods: Light on–1 (70 lx.), Light off (<1 lx.) and Light on–2. Challenges were conducted between 14:00 and 18:00, the most stable activity period in zebrafish larvae (MacPhail et al. 2009). Constant IR lighting was maintained during filming using a three-way switch. The apparatus was enclosed within a lightproof and temperature-controlled box. Distance travelled (cm) was recorded for 200 larvae issued from control fish and 180 larvae issued from exposed fish.

2.9. Data recording and analysis

Videos for the swimming activity and photomotor response done with adults were recorded with an analogue camera ICD-48E (Ikegami) and 2.7–13.5 mm lens (Fujinon) linked to a PC with an acquisition card and Ethovision XT 8.5 software (Noldus, The Netherlands). Videos for the larvae locomotion were recorded using a digital DMK31AU03 camera (The Imaging Sources, Germany) and 1.4–12.5 mm lens (Fujinon) using IC-Capture software (The Imaging Sources, Germany).

For all experiments, EthoVision XT software was used for track extraction and analysis. Data were acquired by EthoVision at 25 frames per second, and variables (distance travelled, time

spent in each area) were nested for further treatments every 30-min for locomotion in adults (24-h), every 1-min in adults and every 30-s in larvae during the photomotor challenge.

2.10. Statistical analysis,

Statistical analyses were realized with Statistica 9.0 (Statsoft, Tulsa, OK, USA) software. Unless otherwise mentioned, the results reported in text and all figures are means ± SEM. General linear model (GLM) was applied in each case.

Growth in length and mass was analyzed with treatments and age as fixed factors, and replicate of origin and fish individuals as random factors. Interaction between treatment and age was evaluated. The normalized number of eggs and survival over 10 days were analyzed with treatment and developmental time (for survival only) as a fixed factor and replicate of origin as a random factor.

For 24-h locomotion activity and photomotor response, distance travelled and vertical positioning were analyzed with periods (day *vs.* night; Light on-1, Light-off and Light on-2) and treatments as fixed factors, replicate of origin, fish individuals as random factors and mass as covariable. Interaction between period and treatment was analyzed.

All statistical analyses were carried out at a 95% level of significance and only fixed factor and interaction are presented in the text. Post hoc tests were with Newman-Keuls in each case.

3. Results

3.1. Sediment, fish exposure and growth

PAHs concentrations measured in spiked sediment are reported in Table 1. Spiking efficiencies for all 3 individual PAHs are in the range [69-77%] (Table 1). Total PAHs concentration was 4407 ± 139 $ng.g^{-1}$ dw for spiked sediment which was more than 600-fold greater than the concentration measured in the control sediment (7.3 ± 1 $ng.g^{-1}$ dw). Sediment particulate organic carbon content was 0.14% dw. Quantification of metabolites indicated a significant increase of 4-OH-Phe, 1-OH-Pyr and 3-OH-BaP in exposed larvae resulting in a six fold increase of the total concentration of hydroxylated metabolites from 585.5 ± 499.2 $ng.g^{-1}$ wet weight to 3801.6 ± 1593.7 $ng.g^{-1}$ wet weight ($p<0.05$; Table 2).

Neither death nor morphological disruptions were recorded during or after embryo-larval exposure with an identical survival rate of 96% between 0 and 4 dpf for both treatments (n=17 triplicates – 51 replicates). Fish body mass was significantly different between control and exposed groups ($F=19.22$; $p<0.001$; Fig. 1a) and post-hoc analysis indicated that body mass of exposed fish was inferior compared to control fish at 5 mpf (control: 0.219 ± 0.004 g; exposed: 0.203 ± 0.006 g; $p<0.05$) and 8 mpf (control: 0.270 ± 0.004 g; exposed: 0.245± 0.007 g; $p<0.001$). At 8 mpf, it was possible to sex the fish unambiguously for both treatments; therefore, body mass was analysed separately for each sex at 8 mpf. In the case of males, there was no difference between treatments (control: 0.270 ± 0.006 g; exposed: 0.255 ± 0.013 g; $p=0.18$) but body mass of exposed females was lower than the one of control females (control: 0.270 ± 0.006 g; exposed: 0.240 ± 0.008 g; $p<0.05$). Body length was significantly different between treatments ($F=8.32$; $p<0.01$; Fig. 1b) and post-hoc analysis revealed a trend for reduction in 5 mpf exposed fish which then became significant

at 8 mpf (control: 2.53 ± 0.02 cm; exposed: 2.46 ± 0.03 cm; $p<0.05$) without difference between sex.

3.2. Reproduction

No difference was observed for spawning onset. The mean number of fertilized eggs per female and spawning request was 2.36 ± 0.4 for control females and 1.42 ± 0.3 eggs for exposed females which was close to a significant difference ($p=0.06$). Offspring developed without significant morphological disruptions whatever the treatment. Survival rates of offspring larvae showed a significant effect of treatment ($F=5.00$; $p<0.05$) and developmental time ($F=13.7$; $p<0.001$) but no interaction between those two factors was observed. Survival rates at hatching (72 hpf) were 80.1 ± 5.6 % and 75.2 ± 6.2 % for offspring of control and exposed parents respectively and these rates decreased respectively to 61.8 ± 8.1 % and 65.0 ± 6.7 % at 10 dpf.

3.3. Locomotion and photomotor responses in top view

Circadian activity was monitored during 24-h (Fig. 2a) and showed the classical alternation of activity during day and night. Comparison of diurnal and nocturnal activities (Fig. 2b) revealed the expected higher diurnal activity for both treatments (period effect in GLM; $F=14.37$; $p<0.01$). Evaluation of photomotor response performed after the 24-h activity monitoring is presented in Fig. 3a. Analysis of the distance travelled for each 15-min periods (Fig. 3b) showed the expected pattern with an increase of activity during Light-off (period effect; $F=77.87$; $p<0.001$). Fish activity was similar for both treatments during the two Light-on periods whereas exposed fish activity was reduced compared to control fish during the Light-off period ($p=0.048$).

3.4. Locomotion and photomotor response in side view

Circadian vertical positioning of fish showed the expected pattern with a preference for the bottom zone during the day compared to the night when fish spent most of the time in the upper zone (Fig. 4). This was revealed by time spent in upper zone which was higher during the night than the day ($F=505.54$; $p<0.001$), the same applied for the complementary variable, time spent in lower zone. A significant difference was also observed for time spent in upper (and lower) zone between treatment ($F=12.90$; $p<0.001$) but there was no interaction between period and treatment. Evaluation of photomotor response performed after the 24-h activity monitoring showed a pattern similar to the one observed over the 24-h: fish spent more time in the top zone during light-off ($F=137.97$; $p<0.001$; Fig. 5). There was however no difference between treatments.

3.5. Photomotor response in F1 5 dpf larvae

Statistical differences were found in periods ($F=211.52$, $p<0.001$) and in interaction between treatment and period ($F=4.46$; $p<0.05$; Fig. 6). The post-hoc test indicated that distance travelled by larvae from exposed fish was higher than distance travelled by larvae from control fish during Light on-2 ($p<0.01$).

4. Discussion

As a sink for most hydrophobic pollutants, including PAHs, sediments often represent a secondary source of pollution for these compounds. This necessitates considering this compartment of the aquatic ecosystem for ecotoxicological assessment. However, direct assessment of sediment toxicity faces some difficulties mainly because of their physico-chemical properties which can interfere with bioassays (for example granulometry or organic matter). In this context, the use of alternate methods has been developed relying on spiking a natural or artificial reference sediment which have both advantages and drawbacks which will not be discussed here (Hollert et al. 2003, Kosmehl et al. 2006, Le Bihanic et al. *Submitted-c,* Vicquelin et al. 2011). In the present study, natural sediment was chosen to evaluate long-term consequences of a sediment-contact exposure during embryonic and first days of larval stages. For this purpose, natural reference sediment was spiked with three individual PAHs abundant in sediment contaminated with PAHs from pyrolytic origin. Concentrations obtained in sediment (sum < 5 $\mu g.g^{-1}$) after spiking was in the range of concentrations measured in contaminated areas (Baumard et al. 1998, Benlahcen et al. 1997, Cachot et al. 2006, Yanagida et al. 2012). Our exposure method can therefore be considered, in terms of compounds, concentration and route of exposure, as a simplified version of environmental exposure.

Quantification of metabolites confirmed an effective exposure to PAHs of larvae and indicated that PAHs metabolism was activated within the duration of exposure. This is in agreement with the early activation of *in vivo* Cyp1a activity in developing zebrafish larvae upon exposure to β-naphtoflavone (Otte et al. 2010) or dioxin (Mattingly & Toscano 2001).

The absence of acute mortality both during exposure and the following days combined with the absence of morphological defects is indicative of no acute toxicity. This is contradictory with results obtained after waterborne exposure to these individual PAHs which provoked, among other phenotypes, oedema, heart and vascular defects (Incardona et al. 2004, Incardona et al. 2006, Incardona et al. 2011). Besides a different exposure route, it is to note that, in order to investigate mechanisms involved in these phenotypes emergence, concentrations used by these authors for BaP, Pyr and Phe were high, in the [5-10 $mg.L^{-1}$] range. Our results are however in agreement with results obtained after embryo-larval exposure of medaka to an artificial sediment spiked with BaP at a similar concentration [1736 $ng.g^{-1}$ dw] (Le Bihanic et al. *Submitted-c*). A delayed effect on survival was however observed after exposure of medaka embryos (10 days of exposure) to a sediment spiked with a pyrolytic extract (Cachot et al. 2007).

Growth inhibition has been reported both after aqueous (Moles & Rice 1983, Schuler et al. 2007) and diet exposure of juveniles fish to PAHs (Gundersen et al. 1996, Jee et al. 2006, Kim et al. 2008, Meador et al. 2006, Vignet et al. In revision, Wang et al. 1993, Wu et al. 2003). Short acute exposure of embryos have also induced reduction of larvae size (Billiard et al. 1999, Incardona et al. 2006, Le Bihanic et al. *Submitted-a,* Le Bihanic et al. *Submitted-b*). To our knowledge, there are however very few reports of delayed effects of an ELS exposure to PAHs on fish growth. A reduction of growth has been reported after a short waterborne exposure of sole juveniles to fuel oil n°2 (Gilliers et al. 2012). In addition, a long-term exposure performed with pink salmon eggs incubated on gravel spiked with Alaska North Slope crude oil showed an early and prolonged growth reduction of larvae and juveniles (Heintz et al. 1999). Because of salmon biology, it is worth mentioning that in this case, exposure lasted 8 months compared to 4 days in our experiment.

PAHs have been shown to affect reproduction in aquatic organisms (Nicolas 1999, Vignet 2014) and several endocrine disruption clues have been reported (Hoffmann & Oris 2006,

Monteiro et al. 2000, Seruto et al. 2005). The late consequences on reproduction of an early exposure are however not known. Here, we described a tendency for a reduction in the number of eggs obtained per female and spawning request, number which still was in the range of what has been described under similar spawning conditions with or without exposure to chemicals (Paull et al. 2008, Roex et al. 2001). Besides possible disruption in reproduction regulation, the decrease observed here may also be related to the reduced growth of exposed female fish. Survival over 10 days was also slightly affected. Further, there are evidences that embryonic exposure to BaP can lead to a reduction of brain aromatase expression (Dong et al. 2008) and this finding provides molecular support for an early endocrine disruption. This particular result should be further investigated.

As a whole, behavioral tests suggested a reduction of locomotor activity and an increase of anxiety in exposed fish. This is in agreement with previous reports of behavioral disruption after exposure to various PAHs. The novelty provided here resides in the exposure itself. Indeed in most cases waterborne exposures have been performed with juvenile stages (Almeida et al. 2012, Correia et al. 2007, Goncalves et al. 2008, Gravato & Guilhermino 2009, Oliveira et al. 2012) and only a few at earlier stages. For example, a waterborne exposure of *Salminus brasiliensis* larvae to phenanthrene produced a reduction of larvae ability to capture prey, in particular when exposure was performed immediately after hatching (Carvalho et al. 2008). Closer to the exposure procedure we used, it has been reported that Pacific herrings eggs exposed to weathered oil showed other disruptions such as a reduction of larvae swimming ability (Carls et al. 1999). Finally, an exposure of medaka embryos by contact with an artificial sediment spiked with benzo[a]anthracene also produced a reduction of larval activity after a photomotor challenge (Le Bihanic et al. *Submitted-d*). Here behavior was evaluated in adults, 6 months after exposure. Our results therefore indicate that early exposure to PAHs is able to promote persistent disruptions. This has already been demonstrated with other compounds such as silver, PBDE or dioxin (Marit & Weber 2012, Powers et al. 2011, Timme-Laragy et al. 2006). From a mechanistic point of view this later case is of particular interest since some PAHs, in particular large one such as BaP, are known to trigger the same molecular pathway as dioxin by activating the aryl hydrocarbon receptor (AhR) (Barron et al. 2004, Carvan III et al. 2000). In this later study, zebrafish larvae were exposed immediately after hatching, from 2 to 4 dpf to dioxin and swimming abilities were assessed 3 months later using a swimming tunnel and both U_{crit} (maximum speed reached before fish exhaustion) and oxygen consumption rate were monitored. For a dioxin concentration of 0.1 $ng.L^{-1}$, the authors reported a decrease of U_{crit} and an increase of oxygen consumption for fish resting and swimming at maximum speed (Marit & Weber 2012). The authors proposed that these effects may be due to behavioral adaptations limiting swimming abilities, failure to mobilize triglyceride stores or vascular deformities limiting blood flow to the periphery (Marit & Weber 2012). Further on this mechanistic approach, monoamines, including serotonin and dopamine are also involved in the control of a wide number of biological processes (see reviews (Hoglund et al. 2005, Overli et al. 2005)) and embryonic disruption of monoamine systems can lead to delayed behavioral disruption (Dennis et al. 2013, Shabanov et al. 2005, Silva et al. 2013). BaP and a prototypic AhR agonist, β-naphtoflavone, were able to disrupt dopaminergic and serotonergic systems in brain of rainbow trout as revealed by an increase of these neurotransmitters turnover (Gesto et al. 2008, Gesto et al. 2009). This provides a molecular framework for delayed behavioral disruption consecutive to an early exposure to PAHs. This hypothesis also fits with the increase of anxiety-like behavior observed in adults zebrafish after an exposure to silver in the same time windows as the one used for PAHs exposure presented here (Powers et al. 2011).

For larvae, it has been proposed that an increase of locomotor activity specially in the outer zone of the well provoked by a "light-to-dark" transition is indicative of anxiety (Schnorr et al. 2012). The authors also indicated that this increase is hardly enhanced by anxiogenic drugs

and they suggest this to be due to a "ceiling effect" which can be bypassed using an incomplete darkness (Schnorr et al. 2012). In our experimental design a total darkness was applied to offspring larvae and therefore we did not observe an increase of activity during the dark period but it was the case in the following light period. The mechanisms underlying the transmission of this anxiety-like behavior to offspring remains unknown and these findings call for more investigations.

Taken together, these observations indicated that an early exposure of fish embryos to a contaminated sediment was able to produce long term consequences on several physiological traits which may have detrimental consequences on their performance and their ability to contribute to recruitment. These results highlight the need to link the findings about PAH contamination in sediments and their effects on fish to the European Water Framework Directive, since the recruitment of information is of high significance for its successful implementation.

Acknowledgements

We thank Didier Leguay (Ifremer, Laboratoire Ressources Halieutiques) for his help. This study was supported financially by the ANR project ConPhyPoP (CES 09_002) and CPER A2E. This later project is co-financed by the European Union with the European fund of regional development. A doctoral grant was received from the Région Poitou-Charentes and from l'Institut Français de Recherche pour l'Exploitation de la Mer (C.V.). This work was part of the LABEX COTE cluster of excellence "Continental to coastal ecosystems".

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Figures

Fig. 1 Evolution of fish body mass (a) and standard length (b) over time between control fish (green bars) and exposed fish (red bars). At 3 mpf n=126 control fish and 74 exposed fish, 123 and 69 at 4 mpf, 77 and 49 at 5 mpf and 71 and 49 at 8 mpf. Values are mean ± SEM; * indicates significant difference at $p < 0.05$ and # indicates significant difference at $p < 0.1$).

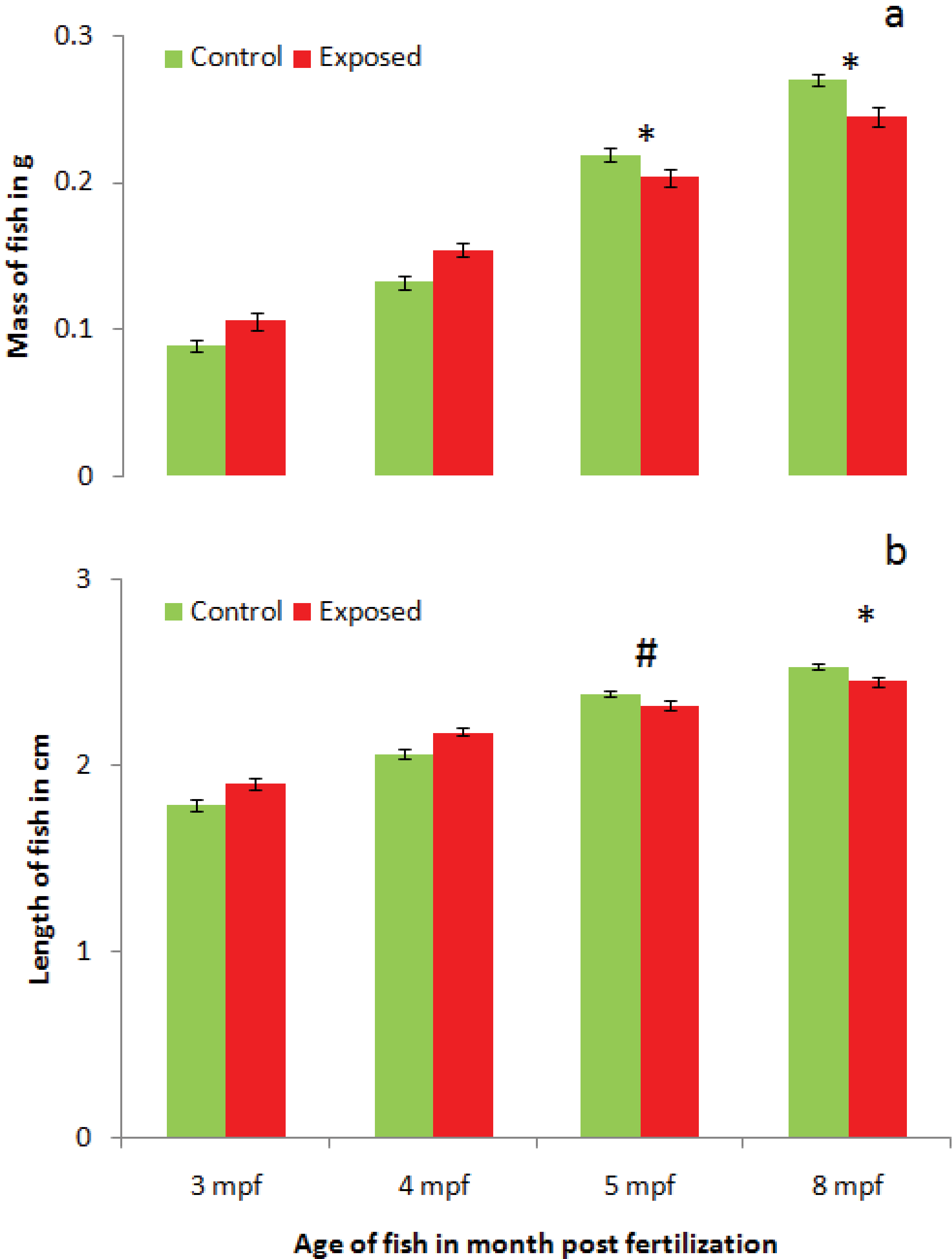


Fig. 2 Horizontal swimming activity of 6 month-old fish in relation to the photoperiod. (a) 24-h evolution of mean distance travelled (cm/30-min) in relation to time of the day and (b) distance travelled averaged per period (day *vs.* night). Values are mean ± SEM; n=36 control and n=24 exposed fish; * indicates significant difference at $p < 0.05$ and # indicates significant difference at $p < 0.1$).

Figure 2

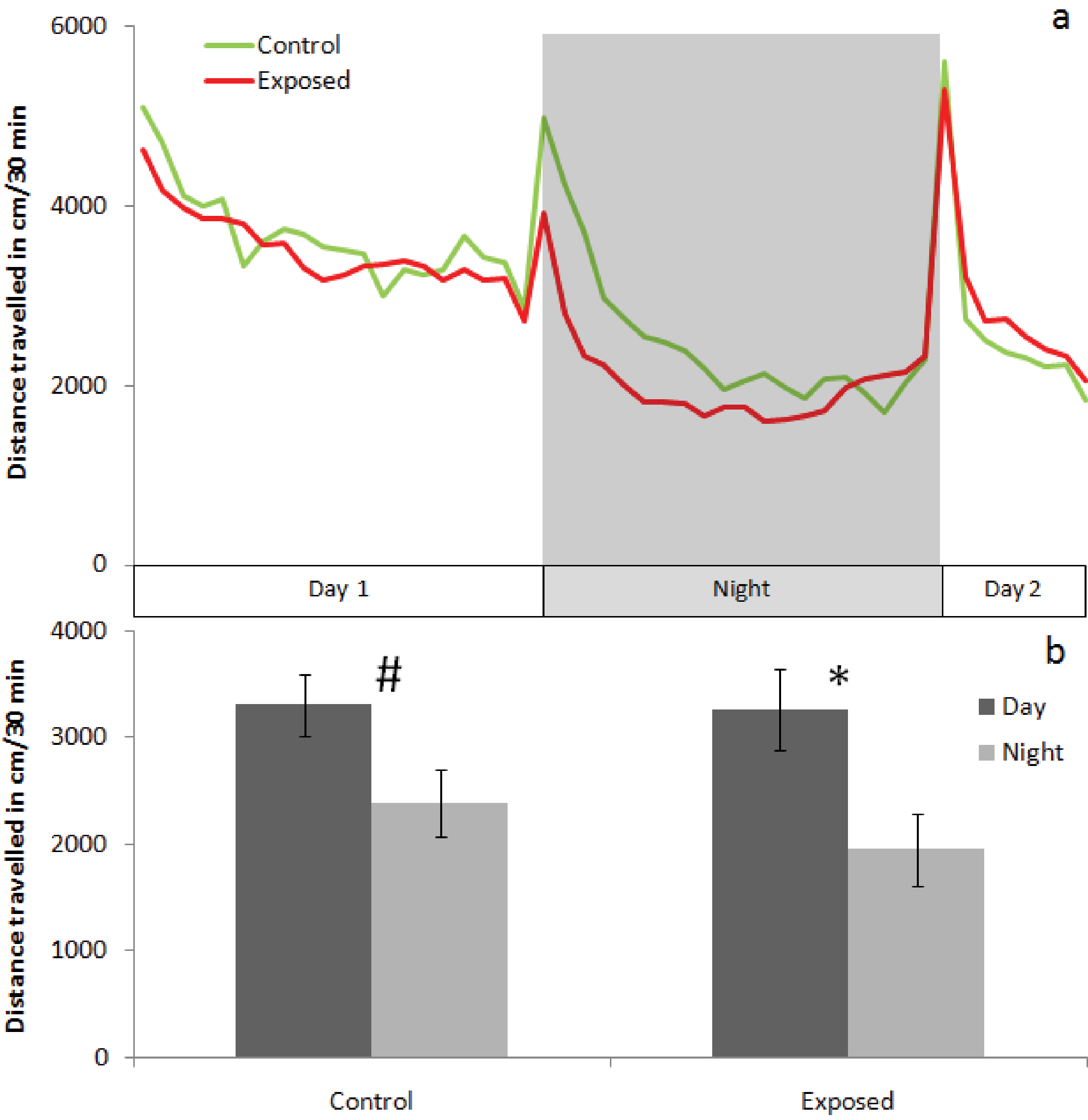


Fig. 3 Photomotor response of 6 month-old fish in top view. (a) Distance travelled recorded during one hour before a 15-min light off period (grey shadowing) followed by an additional 1h30 monitoring. (b) Focus on 15-min periods before, during and after the light off period. Values are mean ± SEM; n=36 control and n=24 exposed fish; * indicates significant difference at $p < 0.05$.

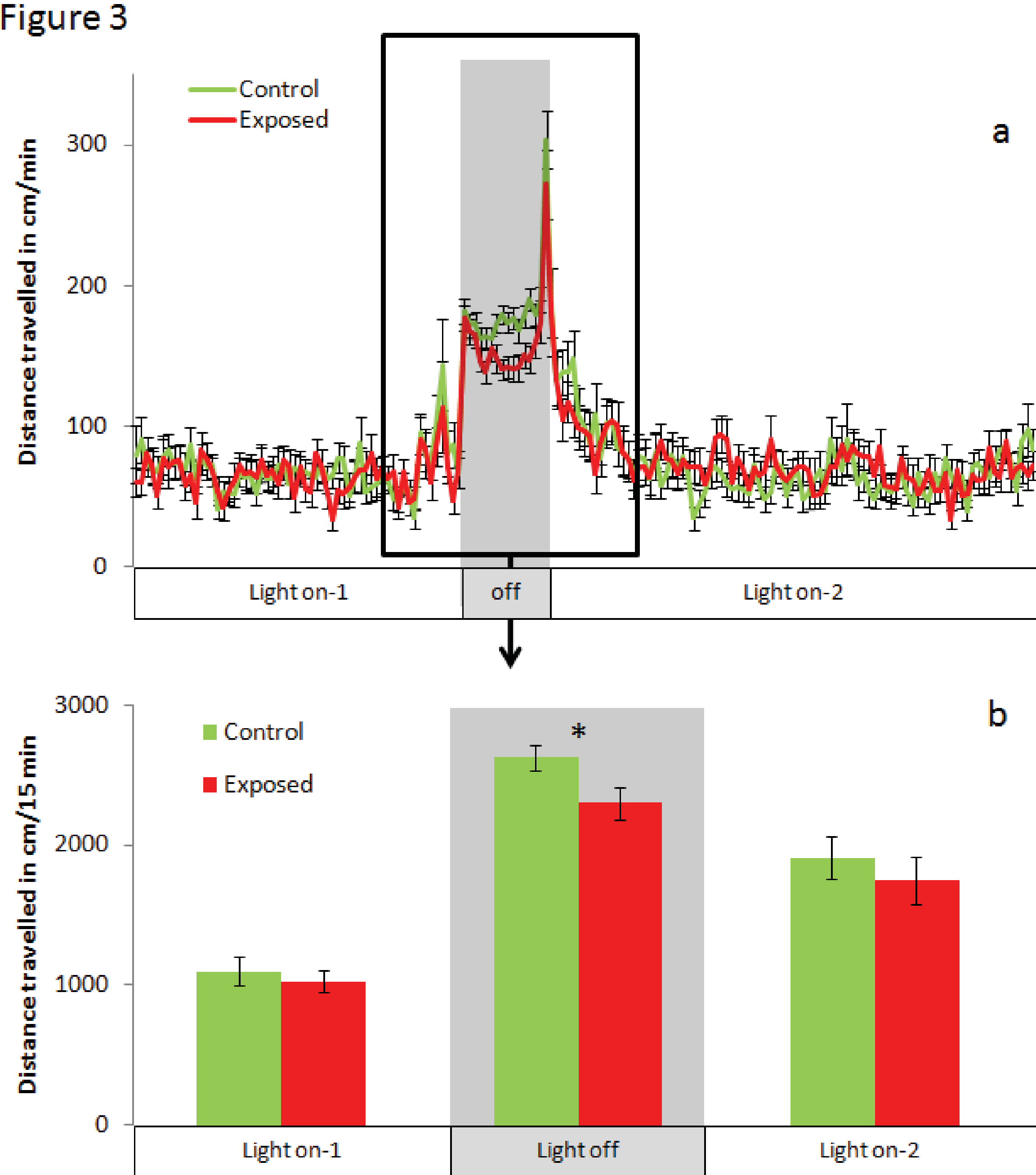


Fig. 4 Vertical swimming position of 6 month-old fish in relation to the photoperiod over 24-h. Evolution of mean time spent (s/30 min) in each third of the tank in relation to time of the day for (a) control fish and (b) exposed fish. Values are mean ± SEM; n=36 control and n=24 exposed fish.

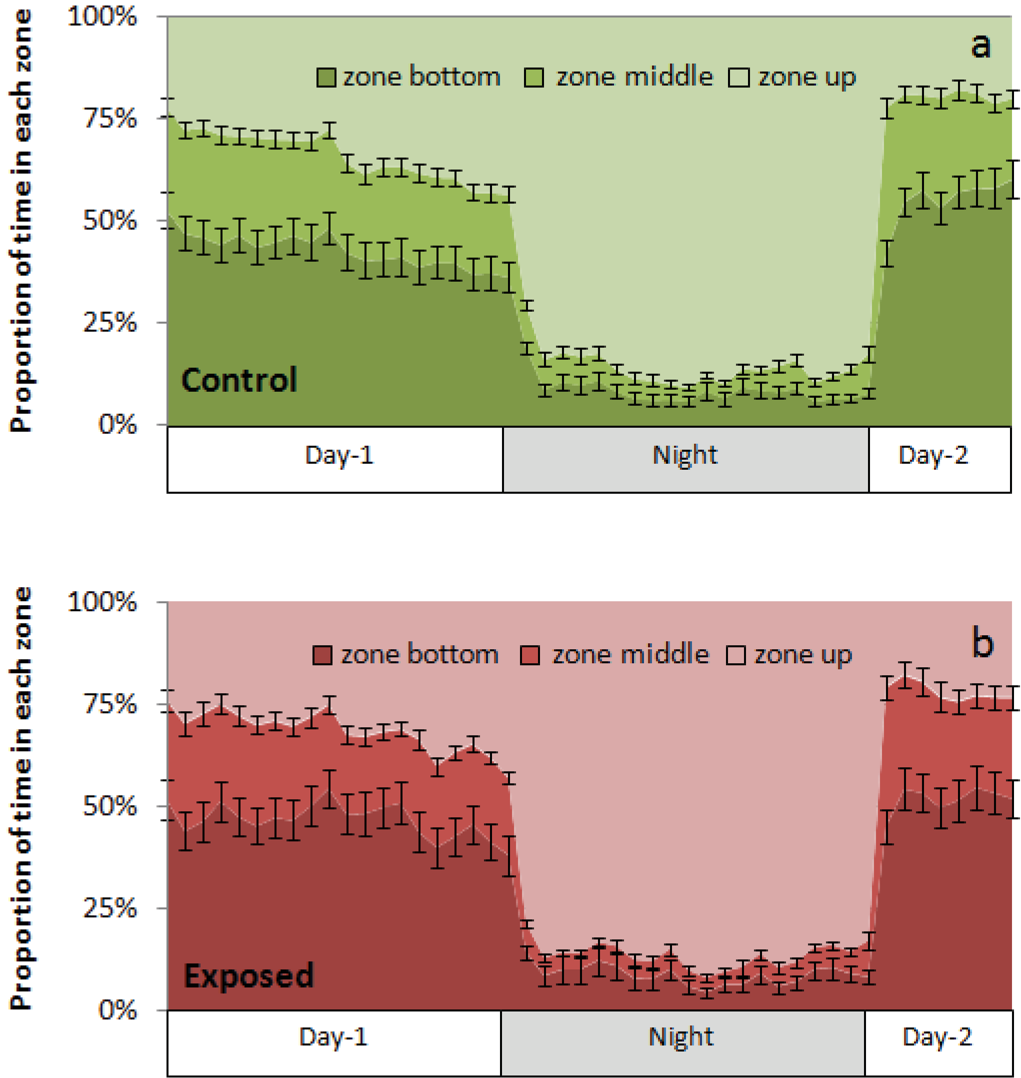


Fig. 5 Photomotor response of 6 month-old fish in side view. Distance travelled per 15-min periods before, during and after the light off period. Values are mean ± SEM; n=36 control and n=24 exposed fish.

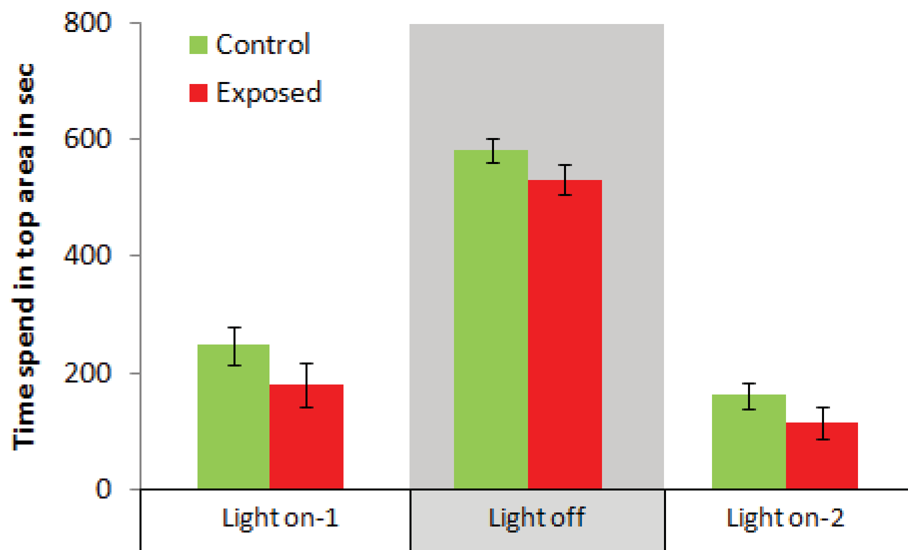
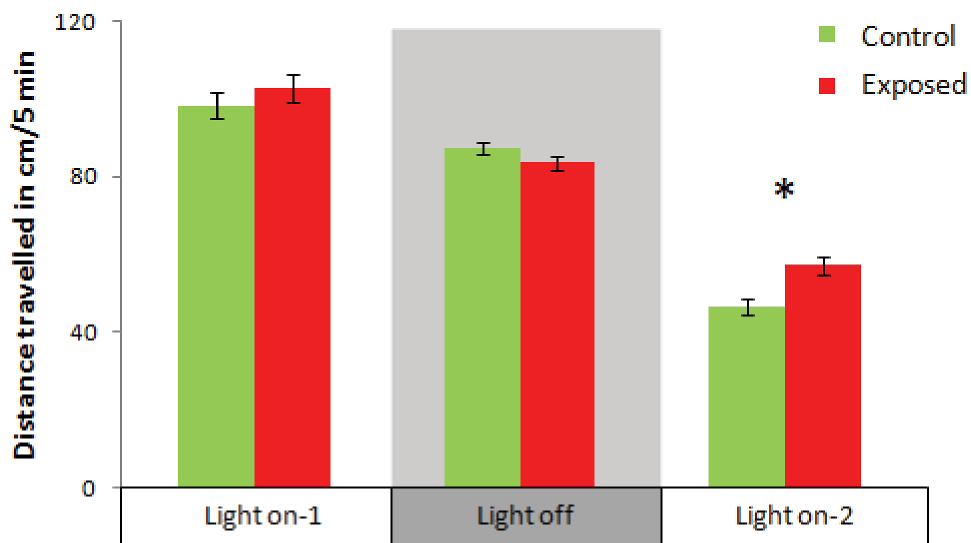


Fig. 6 Photomotor response of 5 dpf offspring larvae. Distance travelled per 5-min periods before, during and after a light off period. Values are mean ± SEM; n=200 larvae issued from control fish and n=180 larvae issued from exposed fish; * indicates significant difference at $p < 0.05$.



Tables

Table 1: Chemical contamination characterization of sediments used

Sediment		Phe	Pyr	BaP
Control	Actual concentration	3.1 ± 0.2	3.5 ± 0.8	0.6 ± 0.1
	Targeted concentration	2750	2350	1250
Spiked	Actual concentration	2126.1 ± 57.6	1648.6 ± 58.9	632.5 ± 32.9
	Spiking efficiency (%)	77	70	51

Concentration are given in $ng.g^{-1}$ dry weight (dw). For actual concentrations values are mean ± SD; n=3 replicates.

Table 2: Metabolites quantification in larvae

	2+3 OH-Phe	1+9 OH-Phe	4 OH-Phe	3OH-Fluo	1 OH-Pyr	1 OH-Chrys	9 OH-BaP	3 OH-BaP	∑ OH-HAP
Control	248.5 ± 218.3	94.1 ± 95.2	<LD	<LD	240.7 ± 193.3	<LD	<LD	<LD	585.5 ± 499.2
Exposed	82.6 ± 38.8	81.8 ± 45	**24.8 ± 13.1**	<LD	**3485.6 ± 1452.9**	<LQ	1.3 ± 0.4	**123.9 ± 49.5**	**3801.6 ± 1593.7**
LD	Sum of OH-Phe: 0.2			0.7	0.7	0.7	Sum of OH-BaP: 0.7		
LQ	Sum of OH-Phe: 0.5			2.0	2.0	2.0	Sum of OH-BaP: 2.0		

Concentration in $ng.g^{-1}$ wet weight; mean ± SD; n=3 replicates of 30 larvae. Numbers in bold indicate significant difference between Control and Exposed larvae ($p<0.05$). LD: detection limit and LQ: quantification limit.