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RESEARCH ARTICLE

Parental stressor exposure simultaneously conveys both adaptive and maladaptive larval phenotypes through epigenetic inheritance in the zebrafish (*Danio rerio*)

Naim M. Bautista* and Warren W. Burggren

ABSTRACT

Genomic modifications occur slowly across generations, whereas short-term epigenetic inheritance of adaptive phenotypes may be immediately beneficial to large numbers of individuals, acting as a bridge for survival when adverse environments occur. In the present study, crude oil was used as an example of an environmental stressor. Adult zebrafish (P_0) were dietarily exposed for 3 weeks to no, low, medium or high concentrations of crude oil. The F_1 offspring obtained from the P_0 groups were then assessed for transgenerational epigenetic transfer of oil-induced phenotypes. The exposure did not alter body length, body and organ mass or condition factor in the P_0 groups. However, the P_0 fecundity of both sexes decreased in proportion to the amount of oil fed. The F_1 larvae from each P_0 were then exposed from 3 hpf to 5 dpf to oil in their ambient water. Remarkably, F_1 larvae derived from oil-exposed parents, when reared in oiled water, showed a 30% enhanced survival compared with controls ($P<0.001$). Unexpectedly, from day 3 to 5 of exposure, F_1 larvae from oil-exposed parents showed poorer survival in clean water (up to 55% decreased survival). Additionally, parental oil exposure induced bradycardia (presumably maladaptive) in F_1 larvae in both clean and oiled water. We conclude that epigenetic transgenerational inheritance can lead to an immediate and simultaneous inheritance of both beneficial and maladaptive traits in a large proportion of the F_1 larvae. The adaptive responses may help fish populations survive when facing transient environmental stressors.

KEY WORDS: Transgenerational inheritance, Crude oil, Epigenetics, Hypoxia, Larva, Heart rate, Environmental stressor

INTRODUCTION

Transgenerational epigenetic inheritance enables parent-to-offspring transfer of modified phenotypes without alteration of the genomic sequence. In its broadest interpretation, this can include maternal/paternal effects (for an introduction into the extensive literature, see Burggren, 2016, 2019; Burggren and Crews, 2014; Heard and Martienssen, 2014; Thorson et al., 2017). Our current understanding of the implications of epigenetic inheritance within the framework of dynamic, stressful environments is limited. Indeed, epigenetically inherited phenotypes have been mostly characterized as maladaptive, especially in human medicine (Baccarelli et al., 2010; Burggren, 2016; Lester et al., 2016; Moosavi and Ardekani, 2016). Unfortunately, this 'maladaptive perspective' of epigenetic inheritance has largely overshadowed the potential role of epigenetic inheritance as a positive mechanism enabling individual animals (and populations) to cope with stressors, and survive and even thrive under short-term environmental challenges (Burggren, 2016). Yet, epigenetic inheritance can also result in the acquisition of adaptive phenotypes that could potentially aid organismal survival (Burggren, 2016, 2014; Laubach et al., 2018; Motta et al., 2015; Vogt, 2017). Such adaptive phenotypes could include improvement of resistance against the stressors experienced by their parents, or even result in increased niche width for the offspring (Herrera et al., 2012; Schrey and Richards, 2012). For example, in the zebrafish (*Danio rerio*), 2–4 weeks of parental exposure to chronic hypoxia confers hypoxic resistance to the F_1 generation (Ho and Burggren, 2012). In killifish (*Fundulus heteroclitus*), F_1 and F_2 embryos from parents inhabiting creosote-polluted sites exposed to creosote contamination showed a lower incidence of cardiac deformities compared with embryos from parents inhabiting non-polluted areas (Clark et al., 2014). Clearly, resistance inherited by the offspring is related to the parental experiences, though the specific mechanisms of epigenetic inheritance have yet to be fully determined. However, experimental protocols exploring epigenetic inheritance are scarce (in part because of their complexity and required time and other resources). Consequently, we still have only a poor understanding of the role of transgenerational epigenetic inheritance during exposure to environmental stressors (Seemann et al., 2017, 2015).

Epigenetic adaptive responses can be generated in response to either natural environmental stressors (e.g. temperature, hypoxia) or anthropogenic stressors [e.g. crude oil and the polycyclic aromatic hydrocarbons (PAHs) it contains]. In fact, the line between natural stressors and anthropogenic stressors is becoming increasingly blurred – consider ambient temperature, for example. Such stressors can have serious consequences for aquatic organisms, and especially fish populations. The actions of these stressors may be through some common pathways, such as the aryl hydrocarbon receptor originally implicated in hydrocarbon-exposure effects (Incardona, 2017), but now also implicated in hypoxia responses (Button et al., 2017; Nie et al., 2001). Exposure to crude oil and the basic cellular and molecular responses it evokes thus represents a contemporary and important environmental challenge. As importantly, the study of the effects of crude oil exposure go beyond toxicology, in fact potentially providing important insights into basic principles behind how individual and population-level survival is affected by numerous environmental stressors, and how epigenetic inheritance may alter survival.

Whether acute or chronic, exposure to crude oil and the thousands of compounds it contains can be a potent environmental stressor. In

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particular, for fish, crude oil exposure may occur via the gills, via diet or by skin contact (Tierney et al., 2013), deeply affecting all developmental stages of fish, from molecular to behavioral levels of organization (Bautista et al., 2019; Brette et al., 2014; Carls et al., 2008; Dubansky et al., 2013; Edmunds et al., 2015; Esbaugh et al., 2016; Frantzen et al., 2012; González-Doncel et al., 2008; Incardona et al., 2004, 2012; Khursighara et al., 2016; Mager et al., 2014; Nelson et al., 2016; Perrichon et al., 2016; Sørhus et al., 2017; Xu et al., 2017a,b). For example, some studies have reported the existence of a link between embryonic exposure to oil and modified phenotypes exhibited during later developmental stages, such as reduced swimming performance and interference with normal heart development (Hicken et al., 2011; Huang et al., 2014; Incardona et al., 2015; Mager et al., 2014). In zebrafish, parental dietary exposure to benzo[a]pyrene, an extensively studied PAH, increased mortality and the presence of body deformities in the F_1 generation, lasting up to the F_3 generation (Corrales et al., 2014). However, the F_1 generation from a parental zebrafish population dietarily exposed to pyrolytic PAHs failed to show significant differences in hatching success or morphological abnormalities, but did exhibit reduced heart rate and differences in yolk sac surface and the ratio of yolk-sac to whole-larval surface (Perrichon et al., 2015). Thus, while the findings of individual studies vary, crude oil and its components can, along with the natural stressors of hypoxia or elevated temperature, serve as a useful 'probe' for exploring transgenerational phenomena and their mechanisms.

Experimentation on the effect on subsequent generations of acute and chronic exposure to natural or anthropogenic stressors is rarely practical with parental wild fish populations. Consequently, the zebrafish has been widely used as a model to perform acute and chronic effect-directed analysis of stressors in several disciplines such as genetics, behavioral sciences, ecotoxicology and physiology (Burggren and Dubansky, 2018; Di Paolo et al., 2015; Jaspers et al., 2014; Milash et al., 2016; Pitt et al., 2018; Spence et al., 2008; Zhou et al., 2019). In the current study, we used the zebrafish, *Danio rerio* (F. Hamilton 1822), to test the hypothesis that dietary crude oil exposure of a parental population will enhance resistance of their larvae (i.e. confer an adaptive phenotype) by means of non-genomic inheritance. To determine whether the parental population per se is affected by dietary exposure to oil, we assessed multiple phenotypic traits in the P_0 generation [i.e. body mass and length, organ mass, condition factor (*K*) and specific growth rate (SGR)] (Barnham and Baxter, 1998; Cook et al., 2000; Williams, 2000). In addition, to better understand the full implications of parental exposure on F_1 survival, we also assessed variables directly related to reproductive success such as fecundity, fertilization, and egg and sperm quality. We also histologically assessed tissue disruption of the gonads and cardiac collagen deposition, which have been associated with oil exposure (Chablais et al., 2011; Gemberling et al., 2013; Grivas et al., 2014; Horn and Trafford, 2016; Kikuchi, 2014; Marro et al., 2016). Having assessed parental effects of oil exposure, we then determined whether the parental exposures transferred epigenetic signals enhancing survival of the offspring. To achieve this, we challenged F_1 fish with oil exposure. In addition, we recorded the heart rate of the F_1 populations during the 5 days of exposure, and determined their growth rate and the presence of edemas and deformities at 5 days post-fertilization (dpf).

MATERIALS AND METHODS

Two separate but complementary experiments were completed during this study. The first, hereafter called the 'fecundity experiment' was performed with 280 zebrafish. Its aim was to determine whether dietary exposure to crude oil affects variables directly related to reproductive success. The second, termed the 'inheritance experiment', was performed with 120 adult zebrafish, and was focused on determining whether parental dietary exposure to crude oil elicits enhanced survival in the F_1 generation during exposure to crude oil via the water. For both experiments, similar protocols, fish care and maintenance, preparation of dietary treatments, parental exposure and F_1 larval exposure were used, unless otherwise specified.

All experiments were approved and performed in compliance with the Institutional Animal Care and Use Committee (IACUC-Protocol #15003) at the University of North Texas.

Fish care and maintenance: parental generation (P_0)

Adult AB strain zebrafish were obtained from a local supplier and maintained individually in 1 l tanks at the University of North Texas. Prior to experimentation, the fish were acclimated for 2 weeks under recommended husbandry conditions for this species (~27±0.5°C, pH ~7.8, 14 h:10 h light:dark cycle, ~7.8 mg l^{-1} dissolved oxygen) (Spence et al., 2008; Westerfield, 2007). Fish were fed ~3% of body mass per day with commercial flake food (TetraMin Tropical food).

Experimental design

Preparation of dietary treatments for P_0 adults

Dietary exposure to crude oil was used as the stressor in experiments with adult zebrafish. To prepare oiled diets, solutions of high energy water accommodated fractions of crude oil (HEWAF) were prepared following standard protocols (Bautista et al., 2019; Forth et al., 2017; Mager et al., 2014; Reddam et al., 2017). Source oil 'B' (SOB) sampled from the Gulf of Mexico MC252 well on 22–23 May 2010 was used for this experiment [British Petroleum acknowledges the use of a defoamer (Nalco EC9323A), oxygen scavenger (Nalco VX9831) and methanol during the collection of this type of crude oil; although their presence in SOB cannot be dismissed, the direct sampling from the riser insertion tube may reduce the possibility of incorporation of these compounds into the oil (de Soysa et al., 2012)]. In brief, 2000 mg of crude oil was added to 1 l of conditioned aquarium water and blended for 30 s in a commercial blender (Waring™ CB15). After blending, the mixture was placed into a separation funnel for 1 h, after which 100 ml of the solution was taken out through a bottom port of the funnel and discarded; 600 ml of the remaining mixture (considered as 100% HEWAF) and two diluted solutions (10% and 50% HEWAF in conditioned aquarium water) were used for diet preparation.

Four dietary treatments were used for parental exposures for adult fish: (1) control, (2) 10% HEWAF, (3) 50% HEWAF and (4) 100% HEWAF. To make these dietary treatments, 2 g of commercial flake food (Tetramin®) was evenly distributed across the bottom of plastic weighing boats (135×135×20 mm L×W×H). The food was sprayed 5 times (5 ml total solution volume) with conditioned water (control) or one of the three HEWAF solution concentrations described above. The spraying process was performed under a fume hood, after which the treated food was allowed to dry for ~12 h. The dried food was then collected from the weighing boats, and stored at 4°C in amber glass bottles covered with aluminium foil.

Representative samples of the treatment diet were analyzed by ALS Environmental (Kelso, WA, USA) to obtain total PAH concentrations. PAHs are petroleum components well known to affect the cardiac system, swimming capacity, performance and morphology throughout development in fish (Incardona et al., 2014; Incardona and Scholz, 2018a; Mager and Grosell, 2011; Stieglitz

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et al., 2016). Thus, determination of PAH concentration in the diets offers a valid indication of the toxicity level of each treatment used during this study (Bautista et al., 2019; Mager et al., 2014). For each food treatment we also measured the sum total of 50 commonly selected PAHs used for the Deepwater Horizon Natural Resource Damage Assessment toxicity testing program (Dubansky et al., 2018; Esbaugh et al., 2016; Johansen et al., 2017; Nelson et al., 2016). Total PAH levels as assayed for each specific dietary composition were proportional to the percentage of HEWAF used to spike the food, indicating the validity of stressor (oil) PAH delivery via this pathway. The control group had a total PAH concentration of less than 0.14 mg kg^{-1} food. Total PAH concentrations of the 10% HEWAF, 50% HEWAF and 100% HEWAF diets were 2.3, 12.8 and 24.2 mg kg^{-1}, respectively (Fig. S1A), and 65–70% of total PAHs for all three diets comprised the 50 selected PAH analytes (Table S1). Unfortunately, estimating the PAH concentration in specific organs was not feasible because of the small size of zebrafish. Thus, ∼12 g of fish (pools of whole animals) per treatment was also sent for analysis. Total alkylated PAH concentrations for each experimental treatment were 15.5, 36.86, 28.3 and 62.82 µg kg^{-1} for females and 10.89, 9.73, 26.2 and 18.29 µg kg^{-1} for males, respectively, for control, 10% HEWAF, 50% HEWAF and 100% HEWAF (Fig. S1B and Table S2).

P_0 crude oil exposure

Adult male and female zebrafish were randomly divided into four groups, each receiving a control diet. After 2 weeks of acclimation to the holding conditions, exposure to petroleum was initiated by feeding the experimental groups the specific diet (control, low, medium or high HEWAF concentration) twice daily over a 3 week period. To prevent possible non-dietary oil exposure through the gills via water, or by coprophagia, during each feeding event, fish were allowed to eat for 10 min, after which non-eaten food and feces were removed. In addition, as the fish were maintained in a closed system, 30% of the water volume of each 1 l tank was also changed after each feeding event (60% per day).

For the fecundity experiment, after the second daily feeding event on day 21 of crude oil exposure, 21 breeding tanks (3 l) per group were established, each containing one female and one male fish from the same parental exposure treatment. The fish were maintained separated by sex overnight. The following morning (day 22) at the start of the light period, in 15 of the tanks the two sexes were placed together for courtship, mating and breeding. Adults in the remaining six tanks were used for histological assessment of the testis and gonadal morphology, and to test sperm motility in the males (see below).

F_1 larvae and crude oil exposure

In the inheritance experiment, after the exposure period, female and male fish from the same parental treatment were placed into 10 l tanks. Fish were also kept separated by sex overnight. The following morning, the fish were allowed to breed for 2 h. The eggs were then collected and rinsed with deionized water and placed in clean conditioned water at 27±0.5°C. Stereoscopic microscopy was employed to confirm fertilization and cell division of the embryos, and any non-viable embryos were discarded.

Crude oil exposures were made on early F_1 larvae from hatching to 5 dpf (Fig. 1), which are among the most sensitive developmental stages (Mager et al., 2017; McKim, 1977; Mohammed, 2013; Réalis-Doyelle et al., 2016; Woltering, 1984). Zebrafish embryos and early larvae subsist on yolk absorbance during the first 5–6 dpf (Anderson et al., 2011; Kimmel et al., 1995), which prevented us from using dietary crude oil exposure as for the P_0 parents. Consequently, oil exposure for the offspring was performed via branchial and cutaneous exposure in ambient water, an exposure equally relevant as that through diet.

F_1 larvae obtained from each parental treatment were subsequently separated into four groups and each group was grown to 5 dpf in one of the following environmental conditions: (1) clean water (control), (2) 10% HEWAF, (3) 50% HEWAF or (4) 100% HEWAF. All larval populations were maintained at 27±0.5°C.

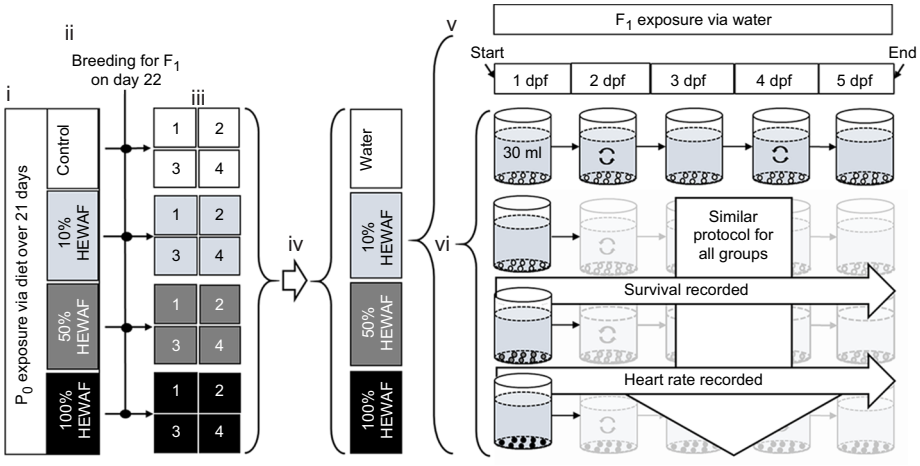

Fig. 1. Experimental protocol. (i) A parental population of adult zebrafish was divided into four groups and exposed via diet to one of the three high energy water accommodated fractions of crude oil (HEWAF) concentrations or to control conditions (water alone) for 21 days. (ii) Offspring (F_1) were obtained from breeding within each of the parental groups on day 22. (iii) The F_1 larvae from each group was subsequently divided into four subgroups, 1–4. (iv) One subgroup from each F_1 group was exposed to water or any of the three HEWAF concentrations for 5 days. For simplification, the F_1 exposure protocol is exemplified by illustrating just the F_1 exposure protocol to 10% HEWAF via water. (v) Exposure to 10% HEWAF began at ∼3 h post-fertilization (hpf) and ended at 5 days post-fertilization (dpf). (vi) The F_1 larvae from the four different parental groups were placed into 50 ml beakers filled with 30 ml of water or one of the three HEWAF solutions. To maintain levels of exposure, the solution was changed on days 2 and 4. Survival and heart rate recordings for all populations were performed throughout the 5 days of HEWAF exposure.

Journal of Experimental Biology (2019) 222, jeb208918. doi:10.1242/jeb.208918

Phenotype measurement

P_0 generation

Adult mortality was assessed daily for each parental treatment. Body mass of individual adults was recorded with a Symmetry EC-Series portable top-loading balance (100 g×0.001 g, 120 V). Individual fish were carefully netted and then immediately placed into a previously tared 100 ml water-filled container to obtain body mass to the nearest mg. The measurement was completed within <30 s. To estimate body length, a lateral photograph of each fish was acquired (Nikon Coolpix AW130, 16 megapixels) during body mass determination, and the measurement was estimated by digital analysis with ImageJ software (https://imagej.nih.gov/ij/). Both body mass and length were measured every second day during the exposure period for the inheritance experiment, and at the end of acclimation and the first, second and third week of exposure for the fecundity experiment.

Body length and mass were used to calculate the specific growth rate (SGR) of each group (Cook et al., 2000). Also determined was the condition factor K (a quantitative index of fish wellness) (Barnham and Baxter, 1998; Williams, 2000), using the formula $K=[(10^5\times M)/L^3]$, where M is the mass of the fish (in g) and L is the length of the fish (in mm).

After breeding, the P_0 adult fish were killed by exposure to a solution of ∼300 mg l^{-1} MS-222 buffered with sodium bicarbonate to pH 7.4. Fish were maintained in the solution for 10 min after opercular movements had ceased, following institutional guidelines. Immediately afterwards, fish were fixed in Z-Fix (Anatech Ltd) for 2 days. The ventricle, liver, gonads and gut were extracted from each fish, weighed and stored in 70% ethanol. Ventricles were processed histologically by first embedding them in paraffin and then sectioning them at 4 µm for staining with Masson's trichrome. This staining technique allowed pixel density assessment by digital analysis to determine whether dietary exposure to crude oil could cause damaging collagen deposition among the extracellular matrix in heart tissue, potentially leading to compromised cardiac activity (Carson, 1990; Huang et al., 2014; Sheehan and Hrapchak, 1980). In brief, photographic images from the heart slices were acquired using a Zeiss Axio Imager.M2 and then analyzed with ImageJ to quantify the area containing collagen. All images were acquired using the same microscopy parameters (scale, zoom, opening of diaphragm). We used gill tissue and bulbus arteriosus slices as a positive control for the stain (see Fig. S4). Based on the staining of these tissues, we determined the color threshold values for blue coloration of collagen (zoom 40×, brightness ratio 150:255, saturation 10:255, and hue ratio 140:190), and used them to standardize the analysis. After setting these parameters in each image, we used the function 'analyze particles' in ImageJ to obtain the number of pixels that met the assumptions for collagen coloration. Six to seven ventricles were analyzed per exposure population, with three different sections per individual analyzed and averaged. Gonadal tissues of both male and female fish were also histologically processed and stained with Hematoxylin and Eosin (H&E). The tissue sections were analyzed by optic microscopy and photographed using the equipment mentioned above.

To test whether fecundity was impaired as a result of crude oil exposure, after allowing the breeding pairs to court and mate for 1 h, the number of parental pairs that spawned was recorded for each experimental group. All the eggs from each breeding pair were carefully collected using a disposable pipette to siphon them from the bottom of the tank. Stereoscopic microscopy was used to determine the total egg number, the number of fertilized eggs, the number of non-fertilized eggs and the number of fertilized but non-viable eggs for each breeding pair.

To assess sperm quality in the fecundity experiment, six breeding pairs from each experimental group (see above) were maintained in overnight conditions as if for breeding, as described above. However, instead of allowing the fish to breed, the next morning, the male fish from each tank were transferred to a specific 3 l container per group. Sperm characteristics were assessed for these adult males following a protocol described elsewhere (Wilson-Leedy and Ingermann, 2007). In brief, males were anesthetized with 100 mg l^{-1} MS-222 solution buffered to pH 7.4. After anesthesia, each fish was carefully netted and dried using a Kimwipe®. Fish sperm become activated when making contact with water, so special attention was paid to drying the area surrounding the male's vent before sampling. After drying, the fish was rinsed in sperm immobilizing medium (ZSI: 140 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 2 mmol l^{-1} $CaCl_2$, 20 mmol l^{-1} Hepes, buffered to pH 8.5 with 1.0 mol l^{-1} NaOH), and then transferred to a sponge previously set for stereoscopic microscopy (Wilson-Leedy and Ingermann, 2007). Fresh seminal fluid was obtained by carefully squeezing the ventral area of the fish and collected by placing a capillary tube in the opening of the vent (Westerfield, 2007). The fish was then placed into a container with aquarium water maintained in recommended conditions (Spence et al., 2008; Westerfield, 2007) and allowed to recover. No mortalities were recorded as a result of this procedure. An average of 1.8 µl of seminal fluid was obtained per fish, from which 1.5 µl was diluted in 10 µl ZSI. Activation of the sperm was attained by diluting 2 µl of the diluted sperm into 10 µl of conditioned aquarium water; 5 µl of this dilution was placed in a depression slide, covered with a coverslip and immediately placed under the microscope for video recording (Zeiss Axio Imager.M2). Three-second videos at 30 frames s^{-1} were recorded for each fish at 100× magnification. The videos were recorded at 20°C and within 20–45 s of sperm activation.

Video analysis was performed using the ImageJ software plugin Computed Assisted Sperm Analysis (CASA; availability and documentation: http://rsb.info.nih.gov/ij/plugins/casa.html). Analyzed variables were: percentage motile sperm; curvilinear velocity (µm s^{-1}), the velocity of the head of the sperm on its curvilinear path; average velocity on a path (µm s^{-1}), the velocity of the head of the sperm along its trajectory; velocity in a straight line (µm s^{-1}), the velocity of the sperm between its initial and final position on a linear path; linearity; and sperm count (Wilson-Leedy and Ingermann, 2007).

Larval F_1 generation: inheritance experiment

Larval mortality experiments were conducted in two phases. In the first phase, the effects of parental HEWAF exposure as a stressor on the survival of F_1 larvae in clean water were determined. The second phase determined the effects of parental HEWAF exposure on the subsequent survival of F_1 larvae when they themselves were exposed to varying concentrations of HEWAF. In both phases of this experiment, mortality (evidenced by the absence of a heart beat) of fish embryos and larvae was assessed on a daily basis from fertilization to 5 dpf.

Heart beat cycles were recorded daily in resting embryos and larvae from each treatment during the 5 days of HEWAF exposure. Heart cycles were videoed over a ∼20 s period using a stereomicroscope (Nikon SMZ1000) adapted with a camera (iPhone 5S). Heart rate (f_H, in beats min^{-1}) was determined in embryos from the video recordings using ImageJ and Adobe PhotoShop CS6 Extended.

This experiment was replicated three times using different adult fish for each replication. With the exception of the subgroups obtained from the parental control group, which were divided into 26 individuals per subgroup for the first replicate, the remainder of

the subgroups and subsequent replicates had a density 50 embryos per 50 ml beaker. Consequently, 16 groups per replicate were obtained in total (Fig. 1).

Larval F_1 generation: fecundity experiment

After determination of fecundity variables mentioned above, the eggs from all parental pairs from the same dietary treatment were mixed. Twenty-five eggs per parental group were placed into a Petri dish containing clean egg water and photographed using a stereoscopic microscope. The area of the chorion and the yolk (mm^2) was estimated for each egg by processing the pictures using ImageJ. Using the area and the radius, the volume of the chorion and the yolk was calculated from the formula of a sphere ($V=¾\pi r^3$) and, in turn, used to calculate yolk to chorion ratio.

From the remainder of the mixed eggs, samples of 50 eggs were taken to recreate the F_1 offspring exposures conditions mentioned above. A total of four beakers per F_1 exposure condition were set for this experiment. Fifteen larvae from two beakers per condition were used to estimate body length at 2 and 5 dpf by image analysis in ImageJ. These measurements were used to estimate SGR. The third and fourth beakers were used to determine the presence or absence of cardiac and yolk edema and/or head and tail deformities at 5 dpf under exposure conditions. Determination of these parameters was performed using stereoscopic microscopy.

Statistical analysis

For both fecundity and inheritance experiments, a three-way ANOVA was conducted for the parental P_0 generation to test whether the level and time of stressor exposure and the sex of the fish induced effects on body mass, body length and condition factor. The Holm–Šidák method was employed to determine pairwise comparisons as *post hoc* tests. SGR in the inheritance experiment was analyzed with one-way ANOVA. Similarly, the mass of the organs and extent of collagen deposition in the heart were compared between dietary treatment groups with one-way ANOVA.

For the fecundity experiment, a χ^2 test was used to compare the proportion of mating pairs that spawned. To assess whether the number of eggs spawned per female was different among the parental groups, analysis of covariance (ANCOVA) was performed using female mass as covariate. One-way ANOVA was used to compare fertilization rate, the number of fertilized eggs, the number of non-viable eggs and the number of non-viable but fertilized eggs among parental groups. Similarly, sperm quality variables were compared among treatments using one-way ANOVA.

Larval F_1 generation

For the inheritance experiment, the survival slopes of the offspring in the different replicates was compared with log-rank survival tests. No differences were found between slope rates ($P>0.05$) within exposure conditions. Thus, we pooled the data of the three replicates and analyzed and plotted them together. We therefore considered n to be 3, where each replicate had 26–50 embryos per condition, as explained above.

To assess the significance of differences in survival rate of F_1 offspring in the inheritance experiment, a Cox stratified model of survival was employed. The F_1 exposure conditions (clean water, 10% HEWAF, 50% HEWAF or 100% HEWAF) were selected as 'strata' in this analysis while the parental exposure background (control, 10% HEWAF, 50% HEWAF or 100% HEWAF) was designated as a covariate. Subsequently, to determine differences between groups within each stratum, survival log-rank tests were employed. Because statistical assumptions of survival analysis do not allow determination of differences between groups at specific points in time, χ^2 tests were performed on each developmental day. Finally, using time (1, 2, 3, 4 and 5 dpf), parental exposure experience and F_1 exposure condition as factors, heart rate of F_1 larvae was compared with a three-way ANOVA.

Assessment of differences in SGR of F_1 larvae from the fecundity experiment was done by two-way ANOVA, in which parental treatment group and F_1 exposure conditions were used as factors.

Finally, to assess, differences in the occurrence of edemas and body deformities among F_1 exposures within parental groups, χ^2 tests were performed.

Statistical significant level was set at $P<0.05$ for all analyses. Data are expressed as means±s.e.m.; unless otherwise indicated, SigmaPlot version 14.0, Statgraphics Centurion version XVI and SPSS version 22 were used to perform the statistical analyses.

RESULTS

Parental P_0 population

Survival

None of the dietary treatments caused any mortality in the P_0 generation during the 21 day time course of the experimental PAH exposures in either the fecundity or inheritance experiments.

Body morphology

From the beginning to the end of the dietary exposure to oil, adult female mean mass increased from 399±21 to 466±21 mg (fecundity experiment) and from 369±39 to 417±42 mg (inheritance experiment). For the fecundity experiment, male body mass was 314.4±16 and 347.6±13 mg at the beginning and end of the exposure, respectively. Male mean mass was 300±14 and 336±12 mg at the beginning and at the end of the exposure period, respectively for the inheritance experiment. Sex was the only factor associated with a significant difference in adult mass in either experiment ($P<0.001$). Neither the level of dietary stressor nor the day of measurement (or their interaction) had any significant effect on adult body mass ($P>0.05$).

In both fecundity and inheritance experiments, experimental time, but not sex or dietary treatment, had a significant effect on adult total body length ($P=0.027$ and $P=0.001$, respectively). No significant interactions between factors were found in either of the experiments ($P>0.05$). For the fecundity experiment, body length increased from 26.3±0.4 to 28.9±0.3 mm in females, and from 27.6±0.5 to 28.307±03 mm in males and fecundity experiments. For the inheritance experiment, female and male mean body length during the oil exposure period increased from 25.8±0.4 to 28.8±0.4 mm, and from 27.8±0.5 to 29.2±0.4 mm, respectively. The condition factor for fish in the fecundity experiments ($K=1.69±0.06$) and the inheritance experiment ($K=-1.56±0.16$) was constant and did not differ among any population throughout the experiments. Similarly, growth rate did not differ between treatment groups (~0.3±0.2% body mass day^{-1}).

In the inheritance experiment, no significant differences were found in the mass of the ventricle, liver and gut (0.21%, 0.39% and 3.42% body mass, respectively) between sexes or between treatments (Fig. 2A). However, gonadal mass was significantly greater in female compared with male adults (10.2±1.1% and 1.5±0.3% body mass, respectively), so data were analyzed separately by sex. In contrast to female gonad mass, which was unaffected by treatment, male gonads differed significantly between treatments ($P=0.035$) (Fig. 2B), although the effects were complex. The gonads were significantly smaller than control (~1.0±0.1% body mass) with the

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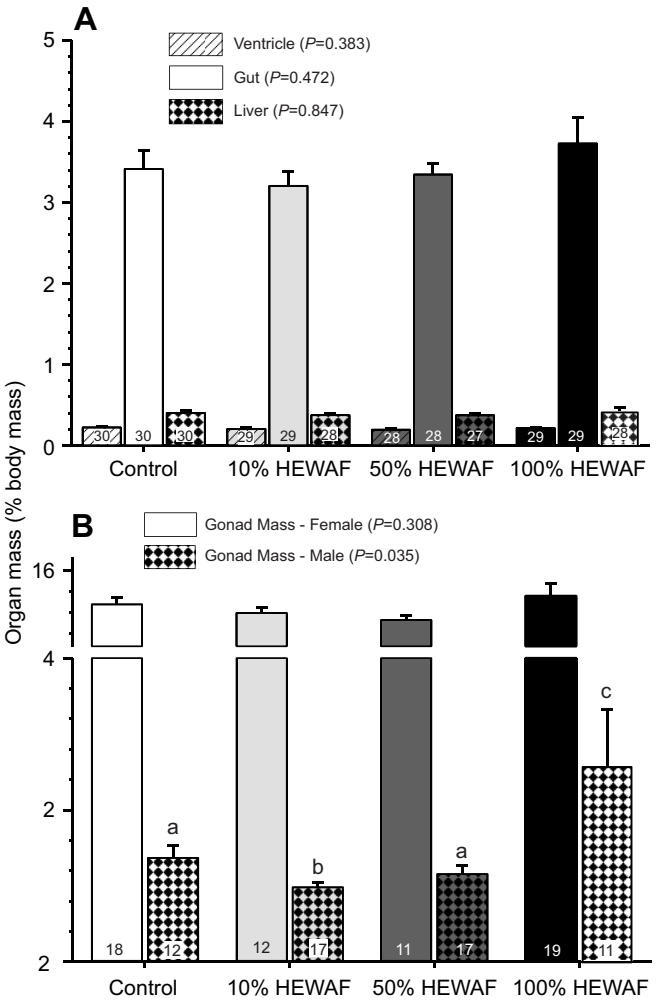

Fig. 2. Effect of crude oil exposure on adult organ mass. (A) Comparison of ventricle, gut and liver mass between treatments (n=27–30 per bar). (B) Female (n=15–18 per bar) and male (n=11–12 per bar) gonadal mass. Organ mass data are given as a percentage of whole body mass (means±s.e.m.). Different letters indicate statistically significant differences between groups (P<0.05).

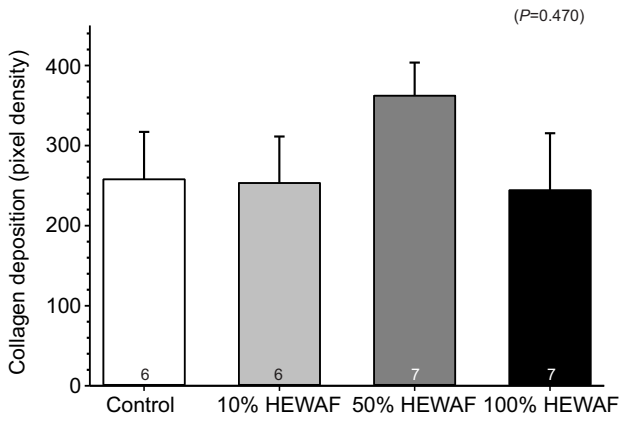

Fig. 3. Effect of crude oil exposure on collagen deposition in adult ventricles. Collagen area is expressed as pixel density obtained from the images of the ventricles from adult zebrafish exposed to different levels of HEWAF. There were no statistically significant differences between treatments (means±s.e.m., P=0.470; n=6–7 per group).

10% HEWAF treatment, but were significantly larger (2.6±0.7% body mass) with the 100% HEWAF treatment.

Upon histological examination, gross morphology of the ventricular tissues appeared to be visually similar in the four groups of P_0 adults (Fig. S4). This observation was confirmed by digital quantification of collagen density in the images, and no significant difference between oil-treated populations was observed (Fig. 3). Similarly, no apparent disruption of gonadal tissue integrity was found in relation to crude oil exposure, as evident from the normal conformation of the lumina, spermatocysts and spermatogonia for male fish (Fig. S2), and the normal conformation of previtellogenic oocytes and vitellogenic oocytes in female gonadal tissue (Fig. S3).

Comparison of egg laying variables among treatments is reported in Table 1. In brief, the total number of eggs laid per female in the higher percentage HEWAF groups was statistically lower than the number laid in the control groups. Similarly, fertilization rates were also lower in the HEWAF groups, and the percentage of non-fertilized and non-viable eggs was greater.

Chorion and yolk volume and the yolk to chorion volume ratio were not significantly different among treatments in the fecundity experiments (Table 2). From the six sperm quality variables (Table 2) estimated from each parental group, only the sperm count per area differed among treatments. In general, all levels of oil exposure reduced sperm count, and in particular sperm count was 50% lower for the highest concentration of crude oil exposure compared with the control group.

Larval F_1 population

Effects of P_0 exposure on F_1 survival

Survival rates of all four of the F_1 larval populations reared in clean water are indicated in Fig. 4A. Parental exposure had a significant effect on the survival rates of their larval offspring when developing in clean water (Cox stratified model, P=0.001). Essentially, F_1 larvae from parents exposed to just 10% HEWAF through diet showed little to no survival differences compared with the F_1 control offspring when developing in clean water. However, parental exposure to 50% or 100% HEWAF resulted in greatly reduced survival rates of the F_1 larvae when developing in clean water, especially from 3 to 5 dpf (log-rank survival test, P<0.001; Fig. 4B).

Parental exposure to 10% HEWAF had no significant effect on mortality of F_1 larvae that were also exposed to 10% HEWAF, at any monitored point in development (Fig. 5A). In part, this lack of significant change resulted from higher variation within the population, with some larvae surviving throughout the developmental period and others succumbing early on.

Reflecting a dose response to crude oil exposure, parental exposure to 50% HEWAF induced significant changes in survival when the F_1 larvae were exposed to the three HEWAF concentrations, especially later in the developmental period (5 dpf) (Fig. 5). There was an interesting dichotomy created by parental exposure levels. F_1 larvae from parents exposed to 10% HEWAF showed improved survival when they themselves were exposed to 50% HEWAF. However, this parentally induced protective effect for larvae in 50% HEWAF was reversed by parental exposure to 50% or 100% HEWAF. Interestingly, during exposure to 100% HEWAF solution, survival rate of F_1 larvae from parents exposed to oil was significantly enhanced in comparison to that of offspring from the control parental group (Fig. 5C).

Essentially, all F_1 larval groups obtained from parents exposed to any level of oil exhibited enhanced resistance to 100% HEWAF throughout the measured developmental period.

Journal of Experimental Biology (2019) 222, jeb208918. doi:10.1242/jeb.208918

Table 1. Fecundity variables resulting from HEWAF exposure in adult male and female zebrafish

	Treatment				
Fecundity variables	Control	10% HEWAF	50% HEWAF	100% HEWAF	*P*-value
n	15	15	15	15	α=0.05
Spawned (yes/no)	15/0^{A}	15/0^{A}	14/1^{A}	7/8^{B}	0.0001
Total no. of eggs	5352	4130	2366	2090	NA
Average no. of eggs per female	356.8±51.7^{A}	275.3±28.8^{A,B}	157.7±23.9^{B,C}	139.3±40.7^{C}	0.002
% Fertilized eggs	75.8±5.4^{A}	38±4.6^{B}	61±5.9^{B}	58.9.±8.1^{B}	0.001
% Non-fertilized eggs	24.2±5.4^{A}	62±4.6^{B}	43.3±7^{A,B}	41.1±8.1^{A,B}	0.001
% Non-viable fertilized eggs	5.9±2.7^{A}	38.1±5^{B}	26.1±7.1^{A,B}	14.6±6.3^{A,B}	0.001

HEWAF, high energy water accommodated fractions of crude oil. Different superscript letters indicate significant differences among dietary treatment groups.

Effects of P_0 exposure on F_1 SGR, edemas and body deformities

Total body length of larvae increased from 3.1±0.02 mm at 2 dpf to 3.9±0.02 mm at 5 dpf. Larval SGR was 3.1±0.01% body length day^{-1}, and did not differ among treatment groups (P>0.05). Neither the parental exposure condition (P>0.05) nor the F_1 exposure condition (P=0.424), or their interaction (P=0.9), had an effect on larvae growth rate.

The presence of edemas and body abnormalities was assessed at 5 dpf in all larval populations. Comparisons between offspring HEWAF exposure conditions within the same parental group are reported in Table 3. When F_1 offspring from control parents were exposed to control conditions, no cardiac or yolk edema, and no head or tail deformities were observed. However, there was a proportional dose-response increase in the percentage of larvae exhibiting those phenotypes when the control larvae were raised in oil conditions. In particular, exposure to 100% HEWAF induced both edema types in 100% of the larvae and more than 80% of them exhibited deformities in their heads or tails. When F_1 offspring from 10% HEWAF-exposed parents were exposed to control and 10% HEWAF, none of the modified phenotypes emerged in the larvae. However, when these larvae were raised in 50% and 100% HEWAF conditions, the percentage of larvae exhibiting edemas or deformities increased proportionally. Importantly, however, the proportion of deformities was smaller than the percentage exhibited by offspring from control parents.

Offspring obtained from parents exposed to 50% and 100% HEWAF exhibited cardiac and yolk edemas when raised in clean water conditions. Neither tail nor head abnormalities were found in offspring from 50% HEWAF parents in clean water, but 40% of the offspring from 100% HEWAF parents exhibited tail abnormalities in this condition. Similarly, when the offspring of these parental groups were exposed to any of the three oil conditions, the F_1 population percentage exhibiting edemas or body deformities was also proportionally increased.

To summarize, exposure to crude oil conditions in F_1 larvae from oil-exposed parents also led to the presence of edemas and body deformities. However, the percentage of the population exhibiting these phenotypes was smaller in F_1 larvae from oil-exposed parents in comparison to the larvae from control parents. These results suggest that parental exposure attenuates adverse effects in their offspring during stressor conditions.

Effects of P_0 exposure on F_1 f_H

Resting f_H in control larvae from control parents was ∼160 beats min^{-1} at 1 and 2 dpf, increasing significantly (P<0.001) to 190–200 beats min^{-1} at 3 and 4 dpf, before declining slightly at 5 dpf to 180 beats min^{-1} (Fig. 6A).

There was a significant interaction between time, parental crude oil exposure and acute F_1 oil exposure via water affecting resting f_H in the F_1 offspring (three-way ANOVA, P=0.001). The patterns of change were complex, however. Parental oil exposure had a marked effect on f_H of F_1 larvae developing in clean water. Essentially, at 3 dpf a depressed f_H (bradycardia) occurred in those F_1 larvae from parents that had been exposed to as little as 10% HEWAF (Fig. 6B). Bradycardias were induced by parental exposure to higher HEWAF levels at this stage of development. Thus, at 1 and 2 dpf, parental exposure to 100% HEWAF led to a larval f_H depression of 50 beats min^{-1}, even when these larvae were raised in clean water. This larval group continued to exhibit bradycardia throughout the 5 days of development in comparison with control-derived offspring.

Table 2. Egg and sperm quality variables in female and male zebrafish exposed to varying HEWAF concentrations

	Treatment				
	Control	10% HEWAF	50% HEWAF	100% HEWAF	*P*-value (α=0.05)
Egg variables					
n	25	25	25	25	
Chorion volume (mm^2)	1.02±0.01	0.95±0.03	0.97±0.02	1.34±0.06	NA
Yolk volume (mm^2)	0.24±0.004	0.24±0.01	0.24±0.01	0.3±0.03	NA
Yolk:chorion volume ratio	0.23±0.004	0.26±0.01	0.25±0.01	0.22±0.01	0.073
Sperm quality variables					
n	6	5	5	6	α=0.05
% Motility	55±4.9	70.8±6.6	74.9±6.5	67±12.3	0.391
Curvilinear velocity	55.7±5.7	57.3±6.7	58.8.±9	78±10.4	0.202
Velocity average path	41.7±2.3	37.1±4.6	46.3±3.8	48.3±3.6	0.165
Velocity straight line	27.1±2.1	23.9±5.8	35.7±1.3	25.8±2.9	0.104
Linearity	66±6.4	60.8±13.5	74.9±6.5	67±12.3	0.145
Count per 0.006 mm^2	18±1.4^{A}	10.8±1.7^{B}	9.4±2.5^{B}	9.3±3^{B}	0.039

Different superscript letters indicate significant differences among dietary treatment groups.

Journal of Experimental Biology (2019) 222, jeb208918. doi:10.1242/jeb.208918

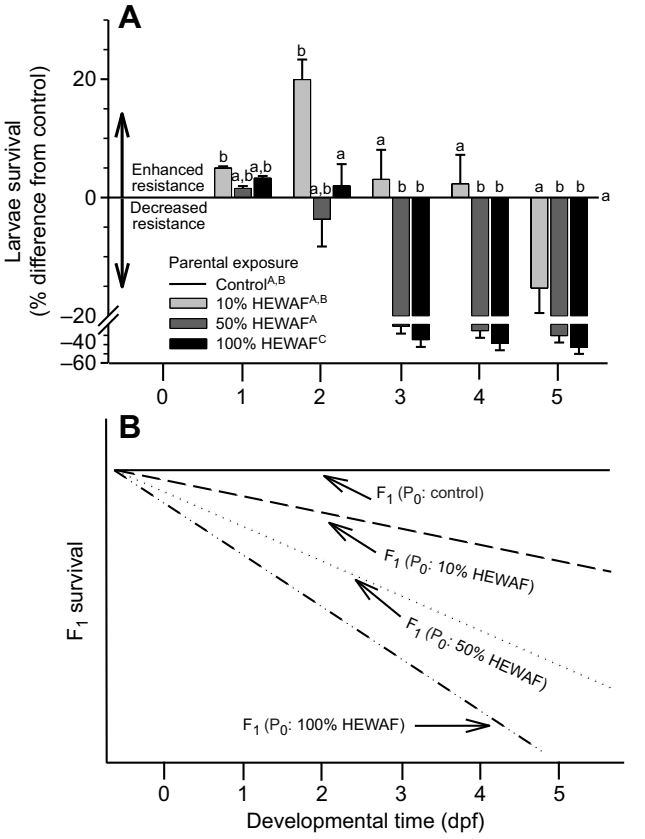

Fig. 4. Effect of parental HEWAF exposure on larval survival. (A) Survival of F_1 larvae raised in clean water. Data are presented as the difference in percentage survival of F_1 larvae from control parents (zero-line) and F_1 larvae from treated parental groups (bars), at a specific developmental time (dpf). Bars above or below the zero-line are interpreted as enhanced or decreased resistance, respectively (data are means±s.e.m., 3 replicates). Different superscript uppercase letters by each parental treatment indicate significant (P<0.001) differences between populations. Different lowercase letters above the bars indicate significant differences between groups at specific times; 'a' was assigned to the control group for all days, and is shown at the end of the zero-line. (B) Schematic representation of survival patterns of F_1 larvae of exposed parents as indicated, raised in clean water, derived from A.

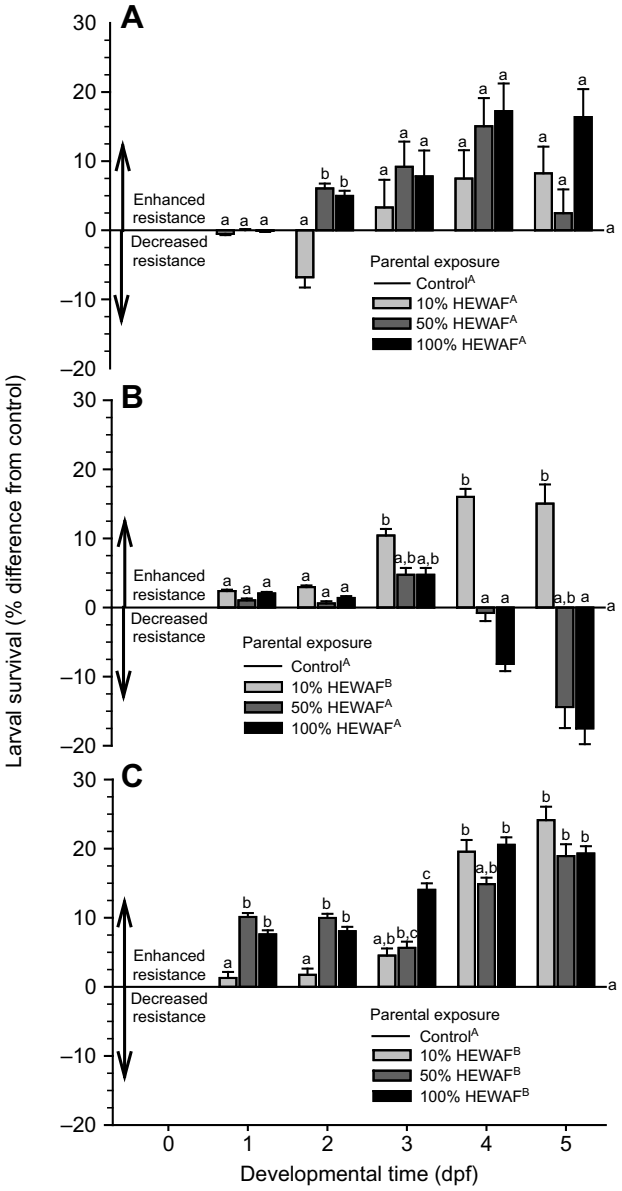

Fig. 5. Synergistic and antagonistic effects of parental and larval HEWAF exposure on larval survival. (A–C) F_1 survival following larval exposure to 10% HEWAF (A), 50% HEWAF (B) and 100% HEWAF (C). Data are presented as the difference in percentage survival between F_1 obtained from control parents (zero-line) and F_1 obtained from treated parental groups (bars), at a specific developmental time (dpf). Bars above or below the zero-line are interpreted as enhanced or decreased resistance, respectively (data are means±s.e.m., 3 replicates). Different superscript uppercase letters by each parental treatment indicate significant (P<0.001) differences between populations. Different lowercase letters above the bars indicate significant differences between groups at specific times; 'a' was assigned to the control group for all days, and is shown at the end of the zero-line. See Results for additional explanation.

Larval offspring obtained from parents exposed to 10% HEWAF showed significant differences in f_H during their early development compared with control-derived offspring (Fig. 6B). At 1 dpf, no differences in f_H occurred between exposure groups (128±3 beats min^{-1}). At 2 dpf, fish exposed to clean water or 10% HEWAF showed a similar f_H of 161±3 beats min^{-1}. However, larval groups exposed to 50% and 100% HEWAF exhibited significantly lower f_H values (143±1 and 124±2beats min^{-1}, P<0.05) in similar conditions. From 3 to 5 dpf, regardless of the exposure concentration of HEWAF, oil-exposed larvae exhibited significant bradycardia (decrease of 50 beats min^{-1}, ∼30%) in comparison to larvae raised in clean water.

Offspring obtained from 50% HEWAF-exposed parents exhibited similar f_H patterns to those obtained from 10% HEWAF-exposed parents at 1 and 2 dpf (Fig. 6C). At 3 dpf, the four larval groups differed from each other (P<0.001), with f_H ranging from 199±3 down to ∼119±6 beats min^{-1}. Although the larvae exposed to 100% HEWAF differed from those treated at all lower concentrations, over the last 2 days all three oil-exposed larval groups showed bradycardia (103±5 beats min^{-1}) in comparison to larvae raised in clean water (164±8 beats min^{-1}).

Finally, exposure to clean water or any of the three HEWAF concentrations had no effect on f_H at 1 dpf (∼117 beats min^{-1}) in offspring obtained from parents exposed to 100% HEWAF (Fig. 6D). At 2 dpf, offspring exposed to clean water and 10% HEWAF showed similar f_H (146±2 beats min^{-1}), which was significantly higher than that of the 50% and 100% HEWAF-treated groups (∼15 beats min^{-1}). From 3 to 5 dpf, the pattern of f_H was similar to that of the larval offspring obtained from 10%

Journal of Experimental Biology (2019) 222, jeb208918. doi:10.1242/jeb.208918

Table 3. Fecundity variables resulting from HEWAF exposure in adult male and female zebrafish

Exposure condition		% Individuals exhibiting abnormalities				SGR (% body length day^{-1})
Parental	F_1 offspring	Cardiac edema (n=15)	Yolk edema (n=15)	Head deformities (n=15)	Tail deformities (n=15)	(n=12–15)
Control	Control	0.00*	0.00*	0.00*	0.00*	3.3±0.1
	10% HEWAF	25.00	8.34*	16.67	16.67	3.2±0.1
	50% HEWAF	46.60	40.00	33.34	26.67	3.3±0.1
	100% HEWAF	100**	100**	83.34**	100**	3.1±0.2
	Pearson χ^2/P-value	25.712/0.0001	34.195/0.0001	23.094/0.0001	33.3/0.0001	
10% HEWAF	Control	0.00*	0.00	0.00	0.00	3.1±0.1
	10% HEWAF	0.00*	0.00	0.00	0.00	3.2±0.1
	50% HEWAF	26.67	26.67	26.67	33.34	3.2±0.1
	100% HEWAF	46.67**	33.34	40**	60**	3.2±0.2
	Pearson χ^2/P-value	15.473/0.001	10.850/0.013	12.960/0.005	21.242/0.0001	
50% HEWAF	Control	13.34	13.34	0.00	0.00	2.9±0.1
	10% HEWAF	20.00	20.00	13.34	20.00	3±0.1
	50% HEWAF	26.67	26.67	26.67	26.67	3±0.1
	100% HEWAF	46.67	40.00	40.00	46.67	3.1±0.1
	Pearson χ^2/P-value	4.773/0.189	3.111/0.375	8.333/0.04	9.317/0.025	
100% HEWAF	Control	26.67	26.67	0.00	40.00	3±0.1
	10% HEWAF	6.67	6.67	6.67	6.67*	2.8±0.1
	50% HEWAF	40.00	20.00	0.00	53.34	3±0.1
	100% HEWAF	60.00	40.00	46.67**	66.67	3.1±0.1
	Pearson χ^2/P-value	10.2/0.017	4.845/0.184	19.615/0.0001	12.274/0.007	

SGR, specific growth rate. Different superscript letters indicate significant differences among dietary treatment groups. Asterisks (* and **, respectively) indicate that the value of the adjusted residuals from the χ^2 analysis was equal to (or beyond) 2 or −2, which deviates them from the H_0 (equal proportions) (Agresti and Kateri, 2011).

HEWAF-exposed parents. All three larval groups exposed to oil exhibited a bradycardia ranging from 135±3 down to 88±6 beats min^{-1} in comparison with those raised in clean water, in which f_H ranged from 186±2 down to 168±7 beats min^{-1} (P<0.05).

f_H effects are summarized in Fig. 7, which shows that a bradycardia resulted from 100% HEWAF exposure at all developmental times and all parental HEWAF exposures.

DISCUSSION

Interest in epigenetic inheritance has burgeoned in the last two decades, and has been largely dominated by the demonstration of the transgenerational transfer of maldapative phenotypes. In contrast, studies focused on demonstrating and interpreting adaptive transgenerational epigenetic inheritance are still relatively scarce (Burggren, 2016; Manjrekar, 2017). Yet, such inheritance could be highly influential in individual- and population-level survival. Consequently, the current study tested whether exposure to a stressor, in the form of parental dietary crude oil, could actually enhance resistance to that stressor in offspring, through non-genomic inheritance.

Parental responses to crude oil exposure

The effects that crude oil and other similar toxicants have on fish have mainly been studied in early developmental stages. However,

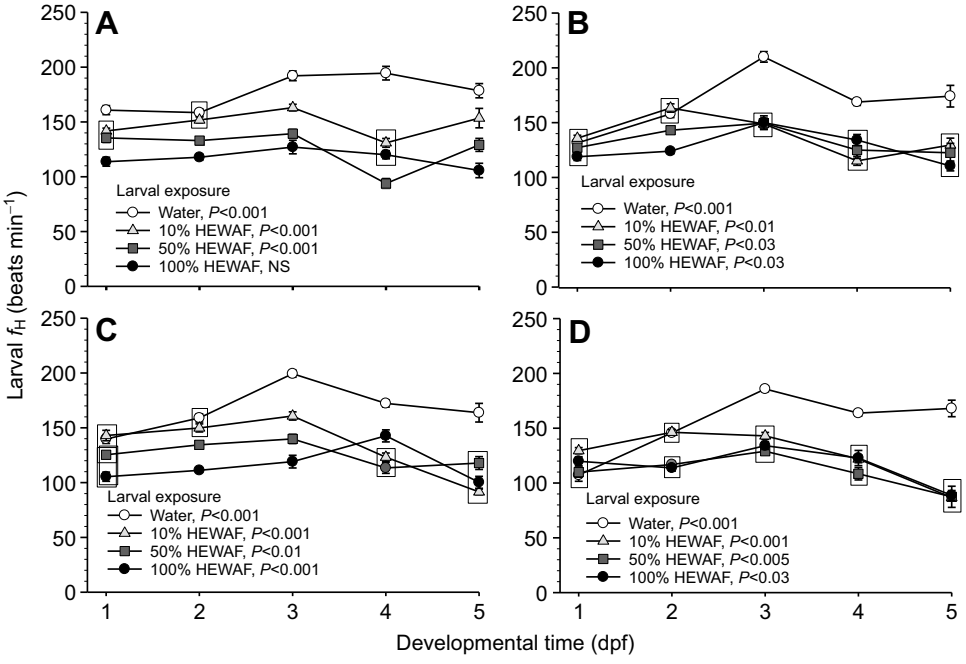

Fig. 6. Heart rate of zebrafish larvae as a function of parental crude oil exposure. (A–D) Heart rate (f_H) of larvae (1–5 dpf) from control parents (A), 10% HEWAF-exposed parents (B), 50% HEWAF-exposed parents (C) and 100% HEWAF-exposed parents (D). Larvae were raised in clean water, 10% HEWAF, 50% HEWAF or 100% HEWAF as indicated. Data are expressed as means±1 s.e.m. Means for any given developmental day that are grouped within boxes are not significantly different (P>0.05). P-values refer to differences across developmental time for each treatment. n=8–74 per data point.

Journal of Experimental Biology (2019) 222, jeb208918. doi:10.1242/jeb.208918

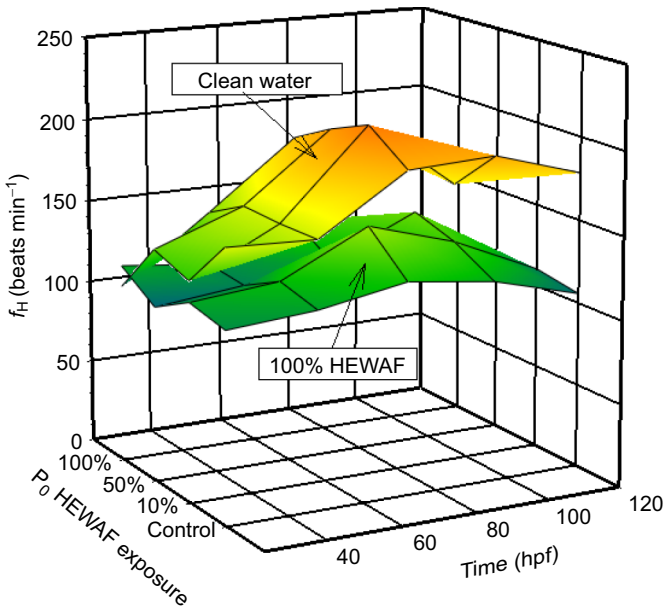

Fig. 7. Comparison of f_H between offspring exposed to clean water (top) and offspring exposed to 100% HEWAF (bottom). Surfaces for 10% and 50% HEWAF exposures are intermediate and have been omitted for clarity.

some studies have evaluated juvenile and adult fish in this context (for review, see Pasparakis et al., 2019). For example, in comparison with controls, 24 h exposure to 8.4 µg l^{-1} of 50 selected PAHs from crude oil induced a 14% decrease in maximum sustained swimming speed (U_{crit}) in young adult mahi-mahi (*Coryphaena hippurus*) (Stieglitz et al., 2016). Similar exposures to 20% HEWAF solution in cobia (*Rachycentron canadum*) induced an 18% increase in f_H, but an offsetting 36% decrease in stroke volume, in oil-exposed fish relative to controls, resulting in no overall change in cardiac output (Nelson et al., 2017).

The present study on adult zebrafish demonstrated that 3 weeks of dietary exposure to sub-lethal concentrations of crude oil-derived HEWAF (0–24.2 mg kg^{-1} food) does not affect survivorship, nor does it compromise primary indicators of adult fish health, such as condition factor or SGR of the parental population. This conclusion is further supported by the lack of effect of oil exposure on the mass of key organs in the P_0 adults, with the exception of male gonads (Fig. 2).

At the tissue level, exposure during early development to oil compounds can induce collagen deposition in the heart of zebrafish at later developmental stages (Huang et al., 2014). Similarly, excessive oil exposure induces collagen build-up in the heart of juvenile salmon (Alderman et al., 2017). However, in the present study on zebrafish, there was no difference in collagen content between the ventricles of the various adult exposure groups (Fig. 3). Similarly, we did not find any indications of gonadal morphological abnormalities for either female or male tissue (Figs S2 and S3). This finding coincides with the reported literature for the polar cod (Bender et al., 2016), where 7 months of dietary exposure still did not induce morphological differences in gonadal tissue. However, in the same study, indicators of sperm viability (curvilinear path velocity, percentage of motile sperm and velocity in a straight line) were affected by exposure. This differs from the findings of the current study, in which only sperm count per area was reduced in fish exposed to any of the three HEWAF conditions (Table 2). This difference may be a function of the different exposure periods.

Parental history and inheritance of adaptive phenotypes in F_1 larvae

Survival of F_1 population

During early development, fish are highly sensitive to multiple stressors – both natural and anthropogenic. Their survival depends on several factors such as length and rate of exposure, and emergent stressors from the interaction of several factors and even parental experiences (Blaxter, 1991; Burggren and Dubansky, 2018; Ehrlich and Muszynski, 1982; Siefert et al., 1973). Hence, offspring phenotypic traits are determined by both genotype and non-genetic contributions of their own or their ancestors' environmental experiences (Auge et al., 2017). However, the ability for offspring to inherit resistance to stressors experienced by the parental population, while potentially adaptive, may also carry trade-offs if these offspring then experience different environmental conditions for which the adaptations leading to resistance may be ill-suited.

Compared with F_1 offspring from control parents, offspring from oil-treated parental groups showed higher survival rates when they themselves were raised in HEWAF conditions (Fig. 5). This is clearly an important adaptation to help survive an adverse environment. At first glance, these results resemble those reported for killifish (Meyer and Di Giulio, 2002, 2003; Ownby David et al., 2009). In those studies, F_1 and F_2 larvae from killifish parents residing in PAH-contaminated areas of the Elizabeth River, VA, USA exhibited increased survival and normal development when exposed to contaminated sediments, when compared with offspring from a reference, non-polluted site. Importantly, however, the experimental design of these studies provided only correlations, and was unable to differentiate between genetic effects, in which the resistance had been selected for in the adult populations, and epigenetic inheritance, in which acute exposure of adults led to the transfer of modified phenotype through an epigenetic marker or another similar mechanism. Indeed, until the present study, the most parsimonious explanation was that the adult killifish had evolved resistance through natural selection, and 'simply' passed this resistance on to their offspring through genetic inheritance. Our studies on zebrafish suggest that there may have been transgenerational epigenetic inheritance in these killifish populations.

Environmental and anthropogenic stressors appear to affect larval stages to a greater extent than they affect embryonic stages (Hutchinson et al., 1998; Mohammed, 2013; Stieglitz et al., 2016). In the present study, differences in the survival rate of the F_1 offspring of control parents, when exposed to clean water or any of the three HEWAF concentrations, were more pronounced from 3 to 5 dpf than at earlier developmental stages (Figs 4 and 5). These results are similar to those of other studies (Perrichon et al., 2016) where, compared with controls, larval zebrafish exposed to water accommodated fractions of heavy fuel oil exhibited decreased survival at 6 dpf compared with earlier developmental stages.

The experimental design of the current study tested the influence of parental experience on offspring survival. Differences in survival rates in the present study were evident only after hatching had occurred (Fig. 4). This could be explained by the fact that the chorion of the embryos may act as an impermeable, or at least partially selective, barrier to crude oil compounds, as it does for the drug amiloride in medaka fish, for example (Cameron and Hunter, 1984). However, there are documented examples of oil-induced changes in embryonic function prior to rupturing of the chorion (Greer et al., 2019; Pasparakis et al., 2016, 2017). An alternative explanation could be that even if dissolved oil components reach the embryo by passing through the chorion, the effects of oil do not become apparent until larval stages in zebrafish; for example, by increasing metabolic demand (Pasparakis

et al., 2017). Additionally, once hatched, larval fishes also face direct exposure to the environment, becoming readily susceptible to phenotypic modification from environmental stressors.

F_1 developmental abnormalities

Exposure to crude oil via water during early development in fish induces cardiac and yolk edema and body abnormalities in a dose–response fashion (Incardona et al., 2014; Incardona and Scholz, 2018b). However, we only poorly understand the effects of parental exposure on larval structure and performance. Our results suggest that 21 days of dietary exposure to crude oil with any of the dietary treatments used in this experiment may attenuate the development of cardiac and yolk edemas and body abnormalities in F_1 offspring during exposure to oil via water.

f_H in the F_1 population

f_H in the zebrafish through all developmental stages is affected by temperature, oxygen availability and anthropogenic toxicants (Barrionuevo et al., 2010; Barrionuevo and Burggren, 1999; Burggren, 2017; Cypher et al., 2017; Horri et al., 2018). In the present study, exposure to crude oil induced bradycardia in control larvae derived from non-exposed parents. These results are similar to those reported for yellow and blue fin tuna and amberjack, where oil exposure created a decrease in heart rate of ∼30%, ∼55% and ∼40% in comparison to control fish, respectively (Incardona et al., 2014). Similarly, oil exposure produced a pronounced bradycardia in embryos of the pacific herring (Incardona and Scholz, 2018b; Incardona et al., 2012), and also decreased f_H, stroke volume and cardiac output in the red drum in a dose-dependent fashion (Khursighara et al., 2016). The general assumption in the literature on fishes is that this persistent bradycardia, opposite to the tachycardia that often occurs in mammals, is maladaptive – or at least not adaptive – especially when accompanied by reduced cardiac output (Perry and Desforges, 2006). However, further experiments are warranted in this regard, as theoretical arguments for an adaptive role for bradycardia have been posited for adult fishes (Farrell, 2007). Moreover, whether bradycardia conveys the same physiological effects in larval and adult fishes is unresolved.

Notably, in the present experiment, major f_H differences between larval groups only developed at 3 dpf. One explanation for this could be that, during the initial development period (<3 dpf), the timing of significant differences in cardiac traits between treatments aligns well with the change from intrinsic to extrinsic factors controlling cardiac function in the zebrafish (Lema et al., 2007; Pelster et al., 2005; Schwerte et al., 2006). Similar to our results, exposure to three-ring PAH compounds (e.g. phenanthrene and dibenzothiophene) did not disrupt the time of onset of heartbeat in zebrafish embryos at 1 dpf, and bradycardia and arrhythmias were present until 3 dpf (Incardona et al., 2004).

Those differences in survivorship and f_H in F_1 offspring were larger after the hatching period, raising questions about the function of the chorion as a protective physical barrier against chemical stressors (see above). Additionally, it is possible that transgenerational maternal provisioning and programming effects could be protecting the embryos until they rely on their own means of protection against stressors (Meyer and Di Giulio, 2003).

Transgenerational epigenetics of F_1 phenotypes

The present study demonstrates that dietary exposure to crude oil extracts, within environmentally relevant concentrations (Vignet et al., 2014), does not affect major indicators of fish health such as condition factor or organ mass of the P_0 adult zebrafish (Figs 2 and 3). However, 21 days of dietary exposure to crude oil did affect male testes mass and sperm count and female egg laying variables. Remarkably, the parental toxicant experience clearly improved the performance of offspring experiencing a similar stressor, as measured by larval survival. One of the most remarkable findings of this study is that when offspring obtained from HEWAF-exposed parents were raised in clean water, their survival actually strongly decreased and they also developed cardiac and yolk edemas (Fig. 4A). In contrast, when offspring obtained from oil-exposed parents were challenged to survive in HEWAF, their survival was significantly greater than those offspring from parents that were not exposed (Fig. 5), and the percentage of them exhibiting edemas was also smaller in comparison with offspring from control parents exposed to the highest HEWAF concentration. A major finding of our study is thus that an adaptive phenotype can be conferred upon offspring through parental exposure to an environmental stressor. Moreover, when combined with the epigenetic inheritance of a bradycardia, we believe this to be the first demonstration of simultaneous inheritance of adaptive as well as maladaptive traits, making for an increasingly complex landscape for epigenetic inheritance.

Potential mechanisms for epigenetic inheritance of larval phenotype

Epigenetically transferred signals from parents to their offspring could induce altered larval gene expression, allowing larvae with temporally low fitness to survive and even exhibit improved resistance against stressors (Burggren, 2016; Ho and Burggren, 2012; Jablonka and Lamb, 2015). It has been shown that resistance to PAHs in subsequent generations was not linked to differences in methylation patterns in CpG sites of the CYP1A promoter (Timme-Laragy et al., 2005), a gene highly involved in detoxification of PAHs (Dubansky et al., 2013; Meyer et al., 2002). These results do not exclude the potential role of other epigenetic mechanisms as complementary means to genetic factors (Nacci et al., 2010) for achieving this end.

Furthermore, as the presence of epigenetic markers varies within a population, it is likely that the genotype frequencies within a population could be also subject to change and indirectly become a substrate for natural selection (Burggren, 2015; Skinner, 2015). As epigenetic inheritance could increase organismal fitness (Klironomos et al., 2013), it has adaptive implications by providing a mechanism for populations to prevail during exposure to anthropogenic stressors (i.e. oil spills, temperature increases) and non-stable natural environments (i.e. seasonal changes in oxygen availability and stochastic temperature fluctuations) (Burggren, 2017, 2019; Burggren and Crews, 2014).

As noted above, transgenerational effects inherited without induction of any change in DNA sequence have received considerable attention during the past two decades (Burggren, 2016; Hu et al., 2018; Inbar-Feigenberg et al., 2013; Jablonka and Raz, 2009). The study of transgenerational epigenetic effects had been linked mostly with maladaptive implications in human-focused disciplines such as medicine (Baccarelli et al., 2010). Consequently, our understanding of the adaptive role of epigenetic inheritance is limited. Studying how epigenetic markers could aid organisms and populations to cope with stressors and prevail under adverse conditions requires implementation of more detailed experiments in which the studied phenotypic variables must embrace a continuum among different levels of organismal organization. In addition, some studies have demonstrated that transgenerational effects that influence offspring phenotypes could arise from both maternal (Nye et al., 2007) and paternal (Lombó et al., 2015) lines.

Conclusions

Our study demonstrates that parental experiences in the form of transient exposure to an environmental stressor prompt a signal transfer to the F_1 generation through non-genomic (i.e. epigenetic) inheritance. The inherited phenotype imbues the F_1 larvae with enhanced survival and attenuation of maladaptive effects when facing similar stressors to those experienced by the P_0 generation. However, our finding that exposure to crude oil during early development induced bradycardia even in offspring obtained from oil-exposed parents indicates that potentially both adaptive and maladaptive traits may be simultaneously inherited through non-genomic means, opening the way for further studies aimed at understanding how populations overcome challenges imposed by changing environments and their stressors. In this sense, experimental design should be directed to test and reveal epigenetic mechanisms involved in gene expression, and the relative contribution of parental experiences on offspring performance. Finally, while crude oil was used as the stressor in this study, we emphasize that these findings may have broad applicability to other stressors, both natural and anthropogenic. Consequently, this type of experiment will provide information for building new foundations and improving our understanding of the transgenerational effects that environmental stressors (e.g. algal blooms causing hypoxia, weather events creating hypothermia or hyperthermia, anthropogenic events such as oil spills) can have on natural animal populations, as well as the repercussions for the survival and prevalence of the species.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.M.B., W.W.B.; Methodology: N.M.B.; Software: N.M.B.; Formal analysis: N.M.B., W.W.B.; Investigation: N.M.B., W.W.B.; Resources: W.W.B.; Writing - original draft: N.M.B.; Writing - review & editing: N.M.B., W.W.B.; Visualization: N.M.B.; Supervision: W.W.B.; Project administration: W.W.B.; Funding acquisition: W.W.B.

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Data availability

Data are publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC, https://data.gulfresearchinitiative.org): doi:10.7266/N7KP80K7.

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.208918.supplemental

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Fig. S1. A) Concentration of PAHs in dietary treatments AND . ∑TotPAH is the sum of all the different PAHs compounds found in the diet. ∑PAH50 represents the fifty most common PAHs in the toxicology literature. **B) Concentration of Alkyl PAHs estimated from pooled whole body fish per treatment group.** Empty bars and patterned bars refere to female and male fish respectively.

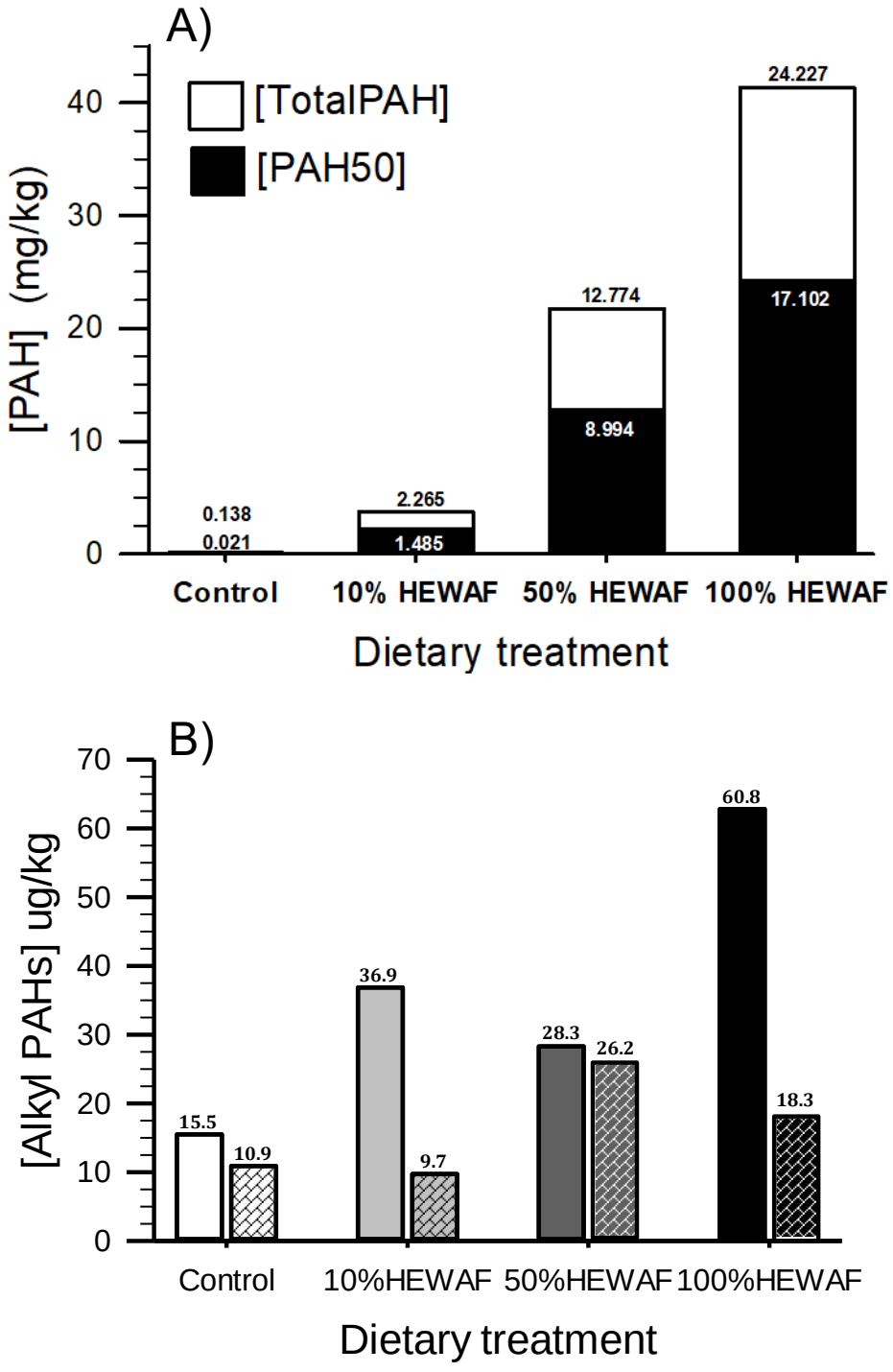

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Fig. S2. Male gonadal sections. A) Control, B) 10%HEWAF, C) 50%HEWAF and D) 100%HEWAF. L= lumina, S= spermatogonia, SP= spermatocysts

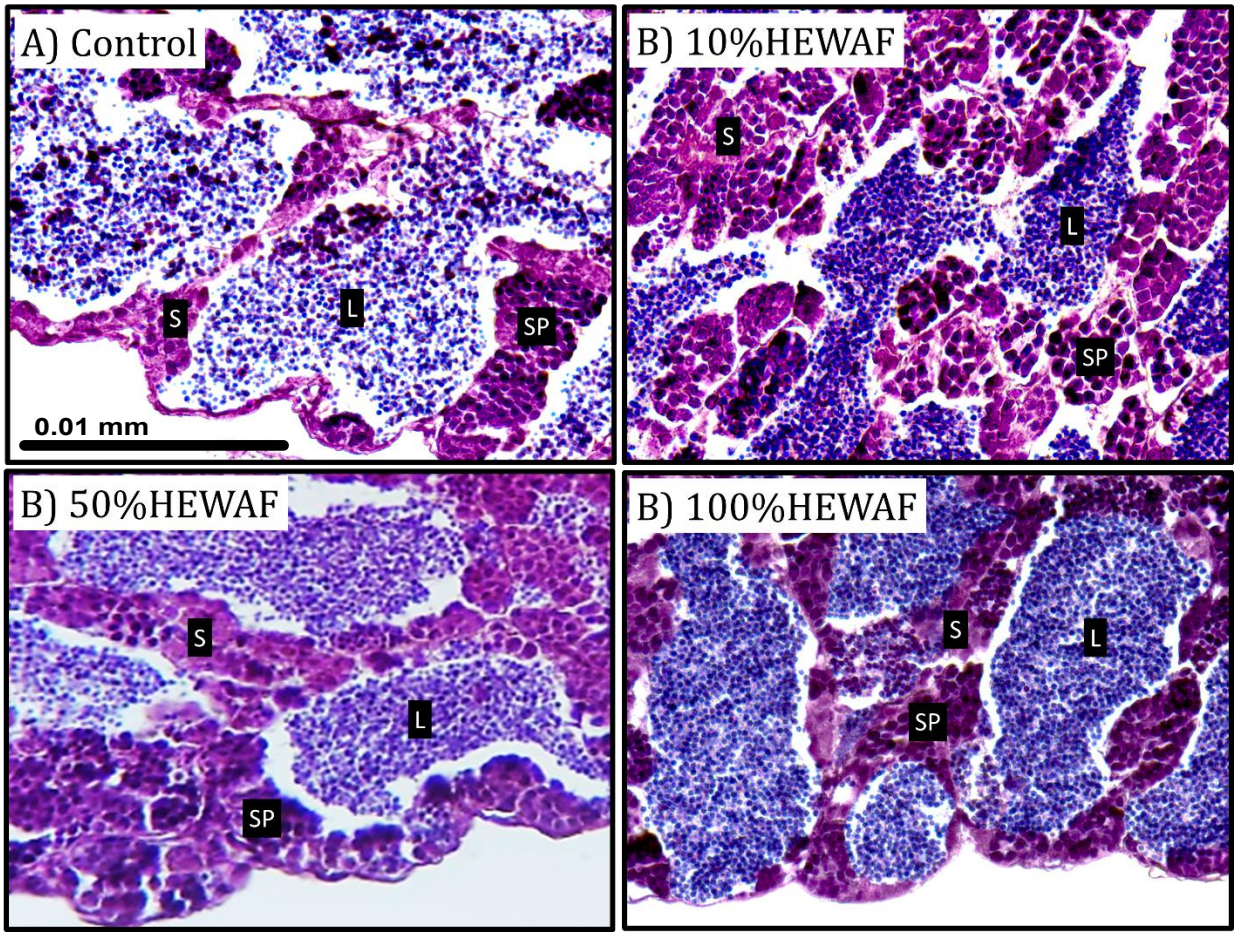

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Fig. S3. Female gonadal sections. A) Control, B) 10%HEWAF, C) 50%HEWAF and D) 100%HEWAF. PO = Previtellogenic oocytes, VO = vitellogenic oocytes.

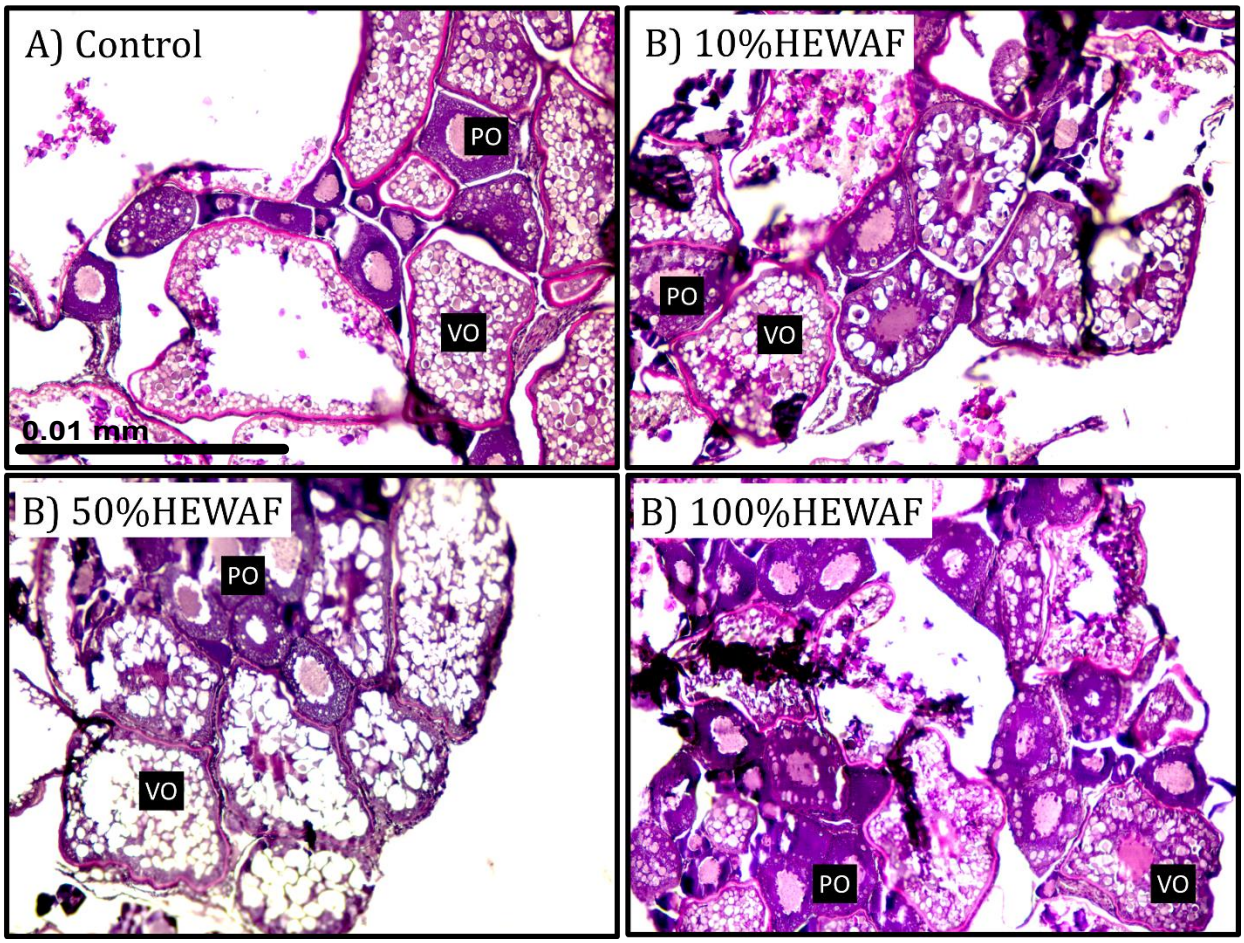

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Fig. S4. Masson's trichrome staining technique in ventricular tissue. Positive control stains, **A)** gill tissue and **B)** bulbus arteriousus tissue. C) Control, D) 10%HEWAF, E) 50%HEWAF and F) 100%HEWAF groups, respectively.

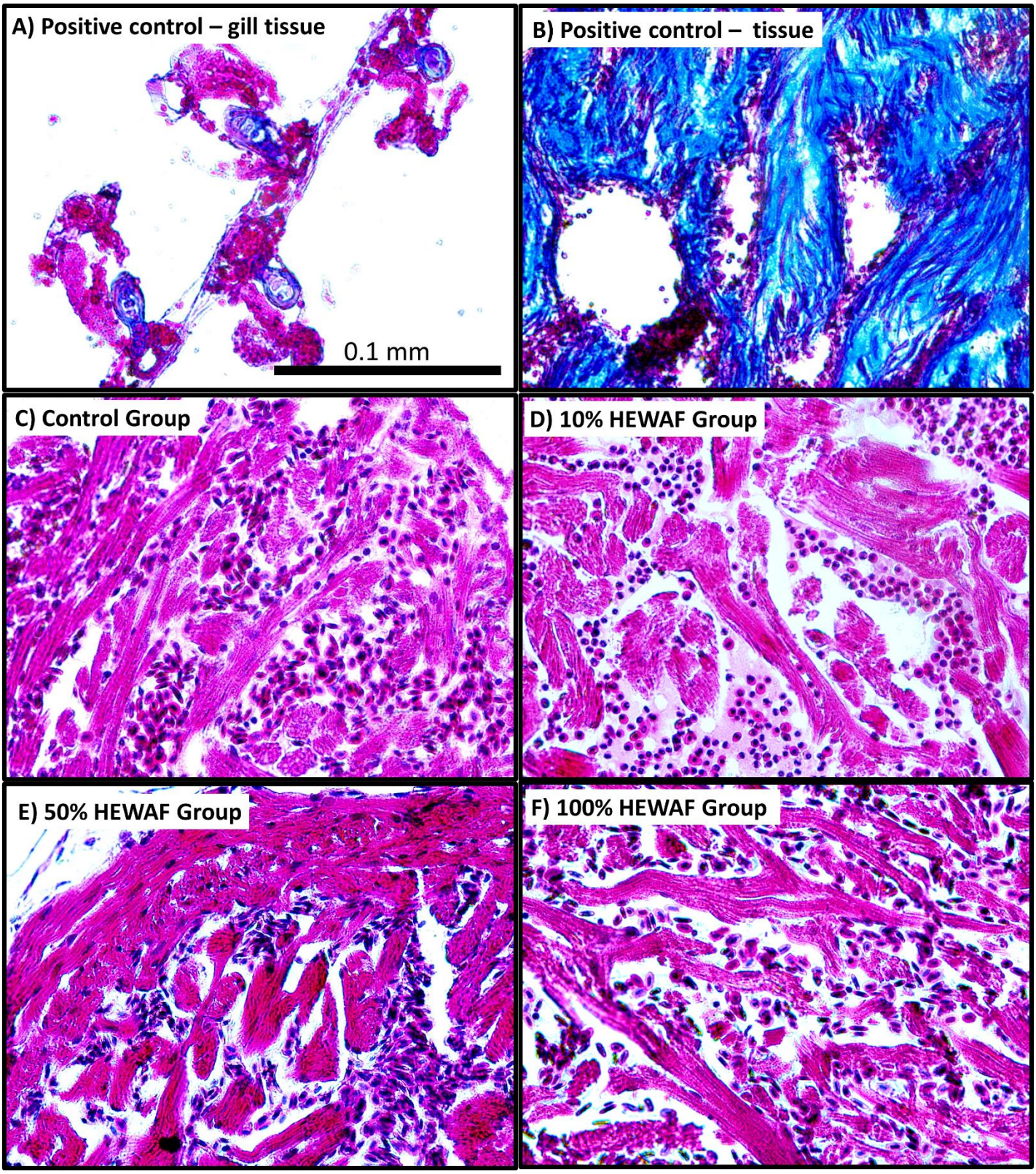

Table. S1. List of components and nominal concentrations (ug/Kg and mg/kg) for each diet treatment. The sum of all the components listed below was considered the "Total PAH concentration". The components highlighted with gray color were considered for the 50 PAHs most frequently measured PAHs (Dubansky et al., 2018; Johansen et al., 2017). ND= not determined.

Polycyclic Aromatic Hydrocarbon (PAHs) Concentrations in dietary treatments					
COMPONENT	CONTROL FOOD (ug/Kg)	10% HEWAF (ug/Kg)	50% HEWAF (ug/Kg)	100% HEWAF (ug/Kg)	BLANK (ug/Kg)
cis/trans-Decalin	117	63.2	72.6	113	ND
C1-Decalins	ND	116	115	212	ND
C2-Decalins	ND	175	269	422	ND
C3-Decalins	ND	ND	412	745	ND
C4-Decalins	ND	ND	518	940	ND
Benzo(b)thiophene	ND	ND	ND	ND	ND
C1-Benzothiophenes	ND	ND	ND	29.3	ND
C2-Benzothiophenes	ND	ND	ND	33.1	ND
C3-Benzothiophenes	ND	ND	ND	41.6	ND
C4-Benzothiophenes	ND	ND	ND	ND	ND
Naphthalene	7.78	36.7	164	312	0.493
C1-Naphthalenes	ND	133	666	1330	ND
C2-Naphthalenes	ND	274	1340	2470	ND
C3-Naphthalenes	ND	245	1270	2140	ND
C4-Naphthalenes	ND	218	781	1310	ND
Biphenyl	ND	19.5	99.6	174	ND
Dibenzofuran	ND	ND	17.0	33.6	ND
Acenaphthylene	ND	ND	D	ND	ND
Acenaphthene	ND	ND	9.28	18.9	ND
Fluorene	ND	18.9	102	206	ND
C1-Fluorenes	ND	53.9	294	563	ND
C2-Fluorenes	ND	126	466	858	ND
C3-Fluorenes	ND	ND	465	810	ND
Anthracene	ND	ND	ND	ND	ND
Phenanthrene	7.16	46.0	232	456	ND
C1-Phenanthrenes/Anthracenes	ND	114	580	1130	ND
C2-Phenanthrenes/Anthracenes	ND	114	686	1260	ND
C3-Phenanthrenes/Anthracenes	ND	85.5	466	908	ND
C4-Phenanthrenes/Anthracenes	ND	ND	249	639	ND
Retene	ND	ND	13.7	22.3	ND
Dibenzothiophene	ND	ND	30.2	55.3	ND
C1-Dibenzothiophenes	ND	ND	112	207	ND
C2-Dibenzothiophenes	ND	ND	163	327	ND
C3-Dibenzothiophenes	ND	ND	109	256	ND
C4-Dibenzothiophenes	ND	ND	ND	ND	ND
Benzo(b)fluorene	ND	ND	9.14	20.0	ND

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Fluoranthene	ND	ND	ND	11.0	ND
Pyrene	ND	ND	20.6	39.2	ND
C1-Fluoranthenes/Pyrenes	ND	ND	67.1	122	ND
C2-Fluoranthenes/Pyrenes	ND	ND	141	255	ND
C3-Fluoranthenes/Pyrenes	ND	ND	136	274	ND
C4-Fluoranthenes/Pyrenes	ND	ND	ND	227	ND
Naphthobenzothiophene	ND	ND	ND	16.4	ND
C1-Naphthobenzothiophenes	ND	ND	ND	85.8	ND
C2-Naphthobenzothiophenes	ND	ND	ND	ND	ND
C3-Naphthobenzothiophenes	ND	ND	ND	ND	ND
C4-Naphthobenzothiophenes	ND	ND	ND	ND	ND
Benz(a)anthracene	6.31	ND	ND	11.6	ND
Chrysene	ND	ND	53.4	77.2	ND
C1-Chrysenes	ND	ND	103	191	ND
C2-Chrysenes	ND	ND	163	294	ND
C3-Chrysenes	ND	ND	ND	ND	ND
C4-Chrysenes	ND	ND	ND	ND	ND
Benzo(b)fluoranthene	ND	ND	ND	ND	ND
Benzo(k)fluoranthene	ND	ND	ND	ND	ND
Benzo(a)fluoranthene	ND	ND	ND	ND	ND
Benzo(e)pyrene	ND	ND	ND	14.7	ND
C30-Hopane	ND	ND	55.5	98.9	ND
Benzo(a)pyrene	ND	ND	ND	ND	ND
Perylene	ND	ND	ND	ND	ND
Indeno(1,2,3-cd)pyrene	ND	ND	ND	ND	ND
Dibenz(a,h)anthracene	ND	ND	ND	ND	ND
Benzo(g,h,i)perylene	ND	ND	ND	ND	ND
4-Methyldibenzothiophene	ND	8.04	48.6	101	ND
2-Methyldibenzothiophene	ND	ND	20.2	39.9	ND
1-Methyldibenzothiophene	ND	ND	16.2	28.8	ND
3-Methylphenanthrene	ND	18.5	101	211	ND
2-Methylphenanthrene	ND	22.4	120	249	ND
2-Methylanthracene	ND	ND	ND	ND	ND
9-Methylphenanthrene	ND	23.8	135	275	ND
1-Methylphenanthrene	ND	21.3	106	207	ND
2-Methylnaphthalene	ND	95.5	506	1010	ND
1-Methylnaphthalene	ND	92.4	491	858	ND
2,6-Dimethylnaphthalene	ND	72.6	467	913	ND
2,3,5-Trimethylnaphthalene	ND	71.3	313	575	ND
Carbazole	ND	ND	ND	ND	ND
Fluorene-d10	82	78	80	73	87
Fluoranthene-d10	94	84	92	83	90
Terphenyl-d14	88	87	95	85	90

	CONTROL FOOD (ug/Kg)	10% HEWAF (ug/Kg)	50% HEWAF (ug/Kg)	100% HEWAF (ug/Kg)	BLANK (ug/Kg)
SUM TOTAL PAH	138.25	2264.54	12774.12	24227.60	0.493

SUM TPAH50	21.25	1484.50	8994.32	17102.70	0.493
	CONTROL FOOD (mg/Kg)	10% HEWAF (mg/Kg)	50% HEWAF (mg/Kg)	100% HEWAF (mg/Kg)	BLANK (mg/Kg)
SUM TOTAL PAH	0.14	2.27	12.77	24.23	0.00
SUM TPAH50	0.02	1.485	8.994	17.10	0.00

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Table. S2. List of components and nominal concentrations (ug/Kg and mg/kg) of for Alkylated PAHs in whole body fish per experimental group. The sum of all the components is listed at the bottom of each column. ND= not determined.

Component	Control Female	Control Male	10%HEWAF Female	10%HEWAF Male	50%HEWAF Female	50%HEWAF Male	100%HEWAF Female	100%HEWAF Male	Blank method
Naphthalene	ND	1.4	1.8	0.95	1.1	ND	1.0	1.2	ND
2-Methylnaphthalene	1.6	ND	2.0	ND	1.4	1.9	2.0	2.3	ND
1-Methylnaphthalene	1.4	ND	2.1	ND	1.3	1.4	1.5	1.8	ND
C2-Naphthalenes	ND	ND	ND	ND	8.1	6.8	15	ND	ND
C3-Naphthalenes	ND	ND	6.7	ND	7.3	8.5	17	ND	ND
C4-Naphthalenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
Biphenyl	ND	ND	ND	ND	ND	ND	ND	2.3	ND
Acenaphthylene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dibenzofuran	1.6	0.79	0.96	ND	ND	ND	ND	0.99	ND
Acenaphthene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fluorene	1.1	1.3	1.9	0.98	1.6	1.1	1.6	1.6	ND
C1-Fluorenes	ND	ND	ND	ND	ND	ND	6.1	ND	ND
C2-Fluorenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C3-Fluorenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dibenzothiophene	ND	ND	1.1	ND	ND	ND	0.92	ND	ND
C1-Dibenzothiophenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C2-Dibenzothiophenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C3-Dibenzothiophenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
Phenanthrene	7.5	6.3	12	7.8	7.5	6.5	9.1	8.1	ND
Anthracene	ND	ND	ND	ND	ND	ND	ND	ND	ND
C1-Phenanthrenes/Anthracenes	ND	ND	7.0	ND	ND	ND	8.6	ND	ND
C2-Phenanthrenes/Anthracenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C3-Phenanthrenes/Anthracenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C4-Phenanthrenes/Anthracenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fluoranthene	1.2	1.1	1.3	ND	ND	ND	ND	ND	ND
Pyrene	0.62	ND	ND	ND	ND	ND	ND	ND	ND

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C1-Fluoranthenes/Pyrenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benz(a)anthracene	0.48	ND	ND	ND	ND	ND	ND	ND	ND
Chrysene	ND	ND	ND	ND	ND	ND	ND	ND	ND
C1-Chrysenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C2-Chrysenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C3-Chrysenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C4-Chrysenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzo(b)fluoranthene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzo(k)fluoranthene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzo(e)pyrene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzo(a)pyrene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Perylene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Indeno(1,2,3-cd)pyrene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dibenz(a,h)anthracene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzo(g,h,i)perylene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fluorene-d10	66	69	66	53	60	53	56	67	68
Fluoranthene-d10	88	88	85	67	76	67	69	85	84
Terphenyl-d14	87	90	87	73	81	73	74	93	81
SUM TOTAL	15.5	10.89	36.86	9.73	28.3	26.2	62.82	18.29	0

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