Parental Stressor Exposure Simultaneously Conveys Both Adaptive and Maladaptive Larval Phenotypes Through Epigenetic Inheritance in the Zebrafish (*Danio rerio*)

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Key words:

Transgenerational Inheritance; Crude oil; Epigenetics; Hypoxia; Larva; Heart rate, Environmental Stressor.

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. Crude oil was used as an example of an environmental stressor. Adult zebrafish (P_0) were dietarily-exposed for three 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. However, when the P_0 were bred, the fecundity in both sexes decreased in proportion to the amount of oil fed. Then the F_1 larvae from each P_0 were exposed from hatch to 5dpf 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 to controls ($P<0.001$). Unexpectedly, from day 3 to 5 of exposure, the 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.

INTRODUCTION

Transgenerational epigenetic inheritance enables parent-to-offspring transference of modified phenotypes without alteration in genomic sequence. In its broadest interpretation, this can include maternal/paternal effects – for an introduction into the extensive literature see (Burggren, 2016; Burggren, 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 acquisition of *adaptive* phenotypes that could potentially aid organismal survival (Burggren, 2016; Burggren, 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 to 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; Seemann et al., 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 PAHs it contains). In fact, the line between natural stressors and anthropogenic stressors is increasingly blurring – 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 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; Incardona et al., 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; Xu et al., 2017b). For example, some studies have reported the existence of a link between embryonic exposures 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). Parental dietary exposure to benzo[a]pyrene, an extensively studied polycyclic aromatic hydrocarbon (PAH), increased mortality and the presence of body deformities in its F_1 generation of zebrafish, 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/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 testing the role on subsequent generations of acute and chronic exposures 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). The current study uses the zebrafish 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 if the parental population *per se* is affected by dietary exposure to oil, we assessed multiple phenotypic traits in the P_O 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 whole 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 if the parental exposures transferred epigenetic signals enhancing survival of their offspring. To achieve this, we challenged F_1 survival with oil exposure. In addition, we recorded the heart rate of the F_1 populations during the 5 days of exposure, and determined growth rate and the presence of edemas and deformities in their body 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 if dietary exposure to crude oil affects variables directly related to their reproductive success. The second, the “inheritance experiment”, was performed with 120 adult zebrafish, and was focused on determining if parental dietary exposure to crude oil elicits enhanced survival in their F_1 generation during exposure to crude oil via water. For both experiments, similar protocols, fish care and maintenance, preparation of dietary treatments, parental exposures, and F_{1} larval exposures 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 two weeks under recommended husbandry conditions for this species (~27±0.5 ºC, pH ~7.8, 14:10h light:dark cycle, ~ 7.8 DO mg/L) (Spence et al., 2008; Westerfield, 2007). Fish were fed ~3% of body weight 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 May 22–23, 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, 2000mg of crude oil were added into 1L of conditioned aquarium water and blended for 30 s in a commercial blender (WaringTM 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: a) Control, b) 10%HEWAF, c) 50%HEWAF, and d) 100%HEWAF. To make these dietary treatments, two g of commercial flake food (Tetramin®) were evenly distributed across the bottom of plastic weighing boats (135L ×135W ×20mmH). The food was sprayed 5 times (5ml 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 hours. The dried food was then collected from the weighing boats, and stored at 4º C in amber glass bottles covered with aluminum foil.

Representative samples of the treatment diet were analyzed by ALS Environmental (ALS Environmental, Kelso, WA, USA) to obtain total polycyclic aromatic hydrocarbons (PAH) concentrations (ΣtotPAH). PAHs are petroleum components well-known to affect the cardiac system, swimming capacity, performance, and morphology throughout developmental stages in fish (Incardona et al., 2014; Incardona and Scholz, 2018a; Mager and Grosell, 2011; Stieglitz et al., 2016). Thus, determination of [PAH] 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). Also measured for each food treatment was the sum total of 50 commonly selected PAHs (Σ50PAH) 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 of food. Total PAH concentrations of 10%HEWAF, 50%HEWAF and 100%HEWAF diets were 2.3, 12.8 and 24.2 mg/kg, respectively (Supplemental Material. Fig. S1.A), and 65-70% of total PAHs for all three diets comprised the 50 selected PAH analytes (Supplemental Material. Table. S1). Unfortunately, estimating the [PAHs] in specific organs was not feasible because of the small size of zebrafish. Thus, ~12g of fish (pools of whole animals) per treatment were also sent for analysis. Total alkylated

[PAH] concentrations for each experimental treatment were 15.5, 36.86, 28.3, and 62.82 ug/Kg for female and 10.89, 9.73, 26.2 and 18.29 for male µg/kg respectively for Control, 10%HEWAF, 50%HEWAF and 100%HEWAF (Supplemental Material. Fig. S1.B 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 two 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 during three 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, since the fish were maintained in a closed system, 30% of water volume of each 1-liter tanks 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 (3L) 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 6 tanks were used for histologically assessing 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 10L tanks. Fish were also kept separated by sex overnight. The following morning, the fish were allowed to breed for 2h. The eggs were then collected and rinsed with deionized water and placed in clean conditioned water at 27±0.5ºC. 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 hatch to 5 days post fertilization (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 days post fertilization (Anderson et al., 2011; Kimmel et al., 1995), which prevented 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 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: a) clean water (control), b) 10%HEWAF, c) 50%HEWAF or, d) 100%HEWAF. All larval populations were maintained at 27±0.5ºC.

Phenotype Measurement

Parental (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 (100g X 0.001g, 120V). 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 sec. To estimate body length, a lateral photograph of each fish was acquired (Nikon Coolpix AW130, 16Mpx) 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 experiment one, and at the end of acclimation, 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) (Barnham and Baxter, 1998; Williams, 2000), using the formula $K = ((10^5 * W) / L^3)$ where; K= condition factor of the fish/quantitative index of fish wellness, W= weight of the fish in grams (g), and L= length of the fish in (mm).

After breeding, the P_0 adult fish were euthanized by exposure to a solution of ~300mg MS-222 /L 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 after euthanasia, fish were fixed in Z-Fix (Anatech

LTD) for two days. The ventricle, liver, gonads and the gut were extracted from each fish, weighed and stored in 70% ethanol. Ventricles of the fish were processed histologically, first embedding them in paraffin, and then sectioning them at 4µm 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 positive control for the stain (See Supplemental material Fig. S4.). Based on the staining of these tissues, we determined the color threshold values for blue coloration of collagen (zoom 40X, 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 the ImageJ software to obtain the number of pixels that meet 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 under optic microscopy and photographed using the equipment mentioned above.

To test if fecundity was impaired due to 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 by 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, the remaining 6 breeding pairs from each experimental group were set and 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 three-litter container per group. Sperm characteristics were assessed on these adult males following the protocol described elsewhere (Wilson-Leedy and Ingermann, 2007). In brief, males were anesthetized using 100 mg/L MS-222 solution buffered to 7.4 pH. 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 – 140mM NaCl, 10mM KCL, 2mM $CaCl_2$, 20mM HEPES, buffered to pH of 8.5 with 1.0 M 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 procedue. An average of 1.8 µl of seminal fluid was obtained per fish, from which 1.5 µl were diluted in 10 µl of 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 per second were recorded for each fish using 100X magnification. The videos were recorded at 20°C and within 20 to 45 sec after 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: percent of motile sperm; curvilinear velocity (VCL µm/s), velocity of the head of the sperm on its curvilinear path; average velocity on a path (VAP, µm/s), which refers to the velocity of the head of the sperm along its trajectory; velocity in straight line (VSL, µm/s), velocity of sperm between its initial and final position on a linear path; linearity (LIN), 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 was determined on the survival of F_1 larvae in clean water. The second phase determined the effects of parental oil 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 absence of heart beat) of fish embryos and larvae was assessed on a daily basis from fertilization through 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 sec period using a stereomicroscope (Nikon SMZ1000) adapted with a camera (iPhone 5S). Heart rate (f_H), in beats per minute, 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 50ml 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 stereoscopic microscopy. The area of the chorion and the yolk (mm^2) were estimated for each egg by processing the pictures using ImageJ. Using the area and the radius, the volume of the chorion and the yolk were calculated from the formula of the 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 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 5dpf 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 if level and time of stressor exposure and the sex of the fish induced effects on body mass, body length and condition factor. Holm-Sidak method was employed to determine pairwise comparisons as post hoc tests. Specific growth rate in the inheritance experiment was analyzed with One-way ANOVA. Similarly, the mass of the organs and extent of collagen deposition in the heart was compared between dietary treatment-groups with One-way ANOVAs.

For the fecundity experiment, a Chi-square test was used to compare the proportion of mating pairs that spawned. To assess if 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 Analysis of Variance was used to compare for 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 Analysis of Variance.

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. Thus we considered n to be 3, where each replicate had 26 – 50 embryos per condition, as above explained.

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, Chi-square tests were performed at each developmental day. Finally, using time (1, 2, 3, 4 and 5 days post fertilization), parental exposure-experience and F_1 exposure condition as factors, heart rate of F_1 larvae was compared with a three-way Analysis of Variance.

Assessment of differences in specific growth rate of F_1 larvae from the fecundity experiment was tested by Two Way Analysis of Variance, in which parental treatment group and the F_1 exposure conditions were used as factors.

Finally, to assess, differences in presence of edemas and body deformities among F_1 exposures within parental groups, Chi-square tests were performed.

Statistical significant level was set at $P<0.05$ for all analyses. Data are expressed as means ± standard error of the mean (SEM), unless other 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_O 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 mg to 466±21 mg (fecundity experiment) and from 369±39 mg to 417±42 mg (inheritance experiment). For the fecundity experiment, male body mass was 314.4±16 mg and 347.6±13 mg at the beginning and end of the exposure, respectively. Male mean mass was 300±14 mg 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 experiments ($P<0.001$). Neither level of dietary stressor nor day of measurement (or their interactions) had any significant effect on adult body mass ($P>0.05$).

In both fecundity and inheritance experiments, experimental time, but not sex nor 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 mm up to 28.9±0.3 mm in females, and from 27.6±0.5 mm 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 mm up to 28.8±0.4 mm and 27.8±0.5 mm to 29.2±0.4 mm, respectively. The condition factor for fish in the fecundity experiments (1.69±0.06) and the inheritance experiment (–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% of body mass/day).

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% of body mass, respectively) between sexes or between treatments (Fig. 2A). However, gonadal mass was significantly larger in female compared to male adults (10.2±1.1% and 1.5±0.3% of 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% of body mass) with 10%HEWAF treatment, but were significantly larger (2.6±0.7% of 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 disruptions 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 (Supplemental Material Fig. S2), and the normal conformation of previtellogenic oocytes and vitellogenic oocytes in female gonadal tissue (Supplemental Material Fig. S3).

Comparison of egg laying variables among treatments are reported in Table 1. In brief, the total number of eggs laid per female in the higher HEWAF% groups were statically lower than the number of the laid eggs in the control groups. Similarly, fertilization rates were also lower in the HEWAF groups, as were the percentages of fertilized and viable eggs.

Volume of the chorion and the yolk and the yolk to chorion volume ratio were not significantly different among treatments in the fecundity experiments (Table 2A). From the six sperm quality variables (Table 2B) 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 in the highest concentration of crude oil exposure compared to 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 lacking any oil exposure, or exposed to just 10% HEWAF through diet, showed little to no survival differences compared to 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 dpf to 5 dpf (Log Rank survival test, $P<0.001$).

Parental exposure to 10%HEWAF had no significant effect on mortality of F_1 larvae also exposed to 10% HEWAF, at any monitored point in the development (Fig. 5A). In part, this lack of significant change resulted from higher variation within the population, with some larvae surviving through 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 day 5 of the developmental period (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 exposures of 50% or 100% HEWAF. Interestingly, during exposure to 100% HEWAF solution, F_1 larvae survival rate from parents exposed to oil was significantly enhanced in comparison to that of the 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.

Effects of P_0 exposure on F_1 specific growth rate, edemas and body deformities

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

Presence of edemas and body abnormalities were 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 nor yolk edema, or 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 F1 offspring from 10%HEWAF exposed parents were exposed to control and 10%HEWAF, none of the modified phenotypes emerged in the larvae. However, when 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 percent exhibited by offspring from control parents.

Offspring obtained from parents exposed to 50%HEWAF 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 attenuate adverse effects in their offspring during stressor conditions.

Effects of P_0 exposure on F_1 heart rate

Resting heart rate in control larvae from control parents was ~160 bpm on day 1 and 2, increasing significantly ($P<0.001$) to 190-200 bpm on day 3 and 4, before declining slightly on day 5 to 180 bpm (Fig. 6A).

There was a significant interaction between time, parental crude oil-dietary exposure and acute oil F_1 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 3dpf a depressed f_H (bradycardia) occurred in those F_1 larvae from parents who 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 days 1 and 2, parental exposure to 100%HEWAF led to a larval f_H depression of 50 bpm, even when these larvae were raised in clean water. This larval group continued exhibiting bradycardia through 5 days of development in comparison with control-derived offspring.

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

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

Finally, exposure to clean water or any of the three HEWAF concentrations had no effect on f_H at 1dpf (~117 bpm) in offspring obtained from parents exposed to 100%HEWAF (Fig. 6D). At 2 dpf, the offspring exposed to clean water and 10%HEWAF showed similar levels of f_H (146±2bpm), which were significantly higher than the 50% and 100%HEWAF-treated groups (~ 115). From 3 dpf to 5 dpf the pattern of f_H was similar to that of the larval offspring obtained from 10%HEWAF exposed parents. All three larval groups exposed to oil exhibited a bradycardia ranging from 135±3 down to 88±6 bpm in comparison with those raised in clean water, which ranged from 186±2 down to 168±7bpm ($P<0.05$).

Heart rate 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 has tested if exposure to a stressor, in the form of dietary parental crude oil, could actually enhance resistance to that stressor in their 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, some studies have evaluated juvenile and adult fish in this context (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 heart rate, but an offsetting 36% decrease in stroke volume, in oil-exposed fish relative to control, resulting in no overall change in cardiac output (Nelson et al., 2017).

The present study on adult zebrafish has demonstrated that 3 weeks of dietary exposure to sub lethal concentrations of crude oil-derived HEWAF (0 to 24.2 mg/kg food) does not affect survivorship, nor does it compromise primary indicators of adult fish health, such as condition factor or specific growth rate 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 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 exposure groups of adults (Fig.3). Similarly, we did not find any indications of gonadal morphological abnormalities for either female or male tissue (Supplemental material Figs. 2-3). This finding coincides with the reported literature for the polar cod (Bender et al., 2016), where seven 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 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 2B). 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 of exposure, rates 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 contribution 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; Meyer and Di Giulio, 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 in 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, their experimental design 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 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 from control parents, when exposed to clean water or any of the three HEWAF concentrations, were more pronounced from 3 to 5 dpf than earlier developmental stages (Fig. 4-5). These results are similar to 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 to earlier developmental stages.

The experimental design of the current study tested the influence of parental experiences 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; Pasparakis et al., 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 demands (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 exposures 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.

Heart rate in the F_1 population

Heart rate 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 heart rate, 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 heart rate 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 for significant differences in cardiac traits between treatments align 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). Similarly to our results, exposure to three-ring PAHs compounds (e.g. phenanthrene and dibenzothiophene) did not disrupt the time of onset of heartbeat in zebrafish embryos at 1dpf, and bradycardia and arrhythmias were present until 3dpf (Incardona et al., 2004).

Those differences in survivorship and heart rate in F_1 offspring were larger after the hatching period, raising the question 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), did not affect major indicators of fish health such as condition factor or organ mass of the P_0 adult zebrafish (Fig. 2, 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 their 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 higher 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 highest HEWAF concentration. A major finding of our study is thus 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 the larvae with temporally low fitness to survive and even exhibit improved resistance against stressors (Burggren, 2016; Ho and Burggren, 2012; Jablonka and Lamb, 2015). Some studies have shown that resistance to PAHs in subsequent generations did not show 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). Their 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, since 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). Since 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; Burggren, 2019; Burggren and Crews, 2014).

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 prompts a signal transfer to the F_1 generation through non-genomic (i.e. epigenetic) inheritance. The inherited phenotype imbues the 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 none genomic means, opening a window for further studies aimed at understanding how populations overcome challenges imposed by changing environments and their stressors. In this sense, experimental designs should be directed to test and reveal epigenetic mechanisms involved in gene expression, and the relative contributions of parental experiences on offspring performance. Finally, while crude oil has been 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 hypo-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.

Attribution statement

This research was made possible by a grant (# RECOVER 1 SA-15-20) from The Gulf of Mexico Research Initiative. Data are publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC) at https://data.gulfresearchinitiative.org (doi: 10.7266/N7KP80K7)

Declaration of interest:

None

REFERENCES

Agresti, A., Kateri, M., 2011. Categorical data analysis. Springer.

Alderman, S.L., Lin, F., Farrell, A.P., Kennedy, C.J., Gillis, T.E., 2017. Effects of diluted bitumen exposure on juvenile sockeye salmon: from cells to performance. Environmental toxicology and chemistry 36, 354-360.

Anderson, J.L., Carten, J.D., Farber, S.A., 2011. Zebrafish lipid metabolism: from mediating early patterning to the metabolism of dietary fat and cholesterol, Methods in cell biology. Elsevier, 111-141.

Auge, G.A., Leverett, L.D., Edwards, B.R., 2017. Tansley insight Adjusting phenotypes via within- and across- generational plasticity.

Baccarelli, A., Rienstra, M., Benjamin, E.J., 2010. Cardiovascular epigenetics: basic concepts and results from animal and human studies. Circulation. Cardiovascular genetics 3, 567.

Barnham, C., Baxter, A., 1998. Condition factor, K, for salmonid fish. Fisheries Notes, 1-3.

Barrionuevo, W., Fernandes, M., Rocha, O., 2010. Aerobic and anaerobic metabolism for the zebrafish, Danio rerio, reared under normoxic and hypoxic conditions and exposed to acute hypoxia during development. Brazilian Journal of Biology 70, 425-434.

Barrionuevo, W.R., Burggren, W.W., 1999. O2 consumption and heart rate in developing zebrafish (Danio rerio): influence of temperature and ambient O2. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 276, R505-R513.

Bautista, N.M., Pothini, T., Meng, K., Burggren, W.W., 2019. Behavioral consequences of dietary exposure to crude oil extracts in the Siamese fighting fish (Betta splendens). Aquatic Toxicology 207, 34-42.

Bender, M.L., Frantzen, M., Vieweg, I., Falk-Petersen, I.-B., Johnsen, H.K., Rudolfsen, G., Tollefsen, K.E., Dubourg, P., Nahrgang, J., 2016. Effects of chronic dietary petroleum exposure on reproductive development in polar cod (Boreogadus saida). Aquatic Toxicology 180, 196-208.

Blaxter, J.H.S., 1991. The effect of temperature on larval fishes. Netherlands Journal of Zoology 42, 336-357.

Brette, F., Machado, B., Cros, C., Incardona, J.P., Scholz, N.L., Block, B.A., 2014. Crude oil impairs cardiac excitation-contraction coupling in fish. Science 343, 772-776.

Burggren, W., 2016. Epigenetic Inheritance and Its Role in Evolutionary Biology : Re-Evaluation and New Perspectives. Biology 5, 1 - 22.

Burggren, W., Dubansky, B., 2018. Development and Environment. 467.

Burggren, W.W., 2014. Epigenetics as a source of variation in comparative animal physiology–or–Lamarck is lookin'pretty good these days. Journal of Experimental Biology 217, 682-689.

Burggren, W.W., 2015. Dynamics of epigenetic phenomena: intergenerational and intragenerational phenotype 'washout'. Journal of Experimental Biology 218, 80-87.

Burggren, W.W., 2017. Epigenetics in insects: mechanisms, phenotypes and ecological and evolutionary implications, Advances in Insect Physiology. Elsevier, 1-30.

Burggren, W.W., 2019. Inadequacy of typical physiological experimental protocols for investigating consequences of stochastic weather events emerging from global warming. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 316, R318-R322.

Burggren, W.W., Crews, D., 2014. Epigenetics in comparative biology: Why we should pay attention. Integrative and Comparative Biology 54, 7-20.

Button, E.L., Bersten, D.C., Whitelaw, M.L., 2017. HIF has Biff – Crosstalk between HIF1a and the family of bHLH/PAS proteins. Experimental Cell Research 356, 141-145.

Cameron, I.L., Hunter, K.E., 1984. Regulation of the Permeability of the Medaka Fish Embryo Chorion by Exogeneous Sodium and Calcium Ions. 454, 447-454.

Carls, M.G., Holland, L., Larsen, M., Collier, T.K., Scholz, N.L., Incardona, J.P., 2008. Fish embryos are damaged by dissolved PAHs, not oil particles. Aquatic toxicology 88, 121-127.

Carson, F., 1990. Histotechnology: A Self-Instructional Text, 1990. ASCP, ILL, 147-149.

Chablais, F., Veit, J., Rainer, G., Ja, A., 2011. The zebrafish heart regenerates after cryoinjury- induced myocardial infarction.

Clark, B.W., Bone, A.J., Di Giulio, R.T., 2014. Resistance to teratogenesis by F1 and F2 embryos of PAH-adapted Fundulus heteroclitus is strongly inherited despite reduced recalcitrance of the AHR pathway. Environmental Science and Pollution Research 21, 13898-13908.

Cook, J.T., Mcniven, M.A., Richardson, G.F., Sutterlin, A.M., 2000. Growth rate , body composition and feed digestibility r conversion of growth-enhanced transgenic Atlantic salmon ž Salmo salar /. 15-32.

Corrales, J., Thornton, C., White, M., Willett, K.L., 2014. Multigenerational effects of benzo [a] pyrene exposure on survival and developmental deformities in zebrafish larvae. Aquatic Toxicology 148, 16-26.

Cypher, A.D., Consiglio, J., Bagatto, B., 2017. Chemosphere Hypoxia exacerbates the cardiotoxic effect of the polycyclic aromatic hydrocarbon , phenanthrene in Danio rerio. Chemosphere 183, 574-581.

de Soysa, T.Y., Ulrich, A., Friedrich, T., Pite, D., Compton, S.L., Ok, D., Bernardos, R.L., Downes, G.B., Hsieh, S., Stein, R., Lagdameo, M.C., Halvorsen, K., Kesich, L.-R., Barresi, M.J.F., 2012. Macondo crude oil from the Deepwater Horizon oil spill disrupts specific developmental processes during zebrafish embryogenesis. BMC Biology 10, 40.

Di Paolo, C., Seiler, T.-B., Keiter, S., Hu, M., Muz, M., Brack, W., Hollert, H., 2015. The value of zebrafish as an integrative model in effect-directed analysis-a review. Environmental Sciences Europe 27, 8.

Dubansky, B., Verbeck, G., Mach, P., Burggren, W., 2018. Methodology for exposing avian embryos to quantified levels of airborne aromatic compounds associated with crude oil spills. Environmental Toxicology and Pharmacology 58, 163-169.

Dubansky, B., Whitehead, A., Miller, J.T., Rice, C.D., Galvez, F., 2013. Multitissue molecular, genomic, and developmental effects of the Deepwater Horizon oil spill on resident Gulf killifish (Fundulus grandis). Environmental Science & Technology 47, 5074-5082.

Edmunds, R.C., Gill, J.A., Baldwin, D.H., Linbo, T.L., French, B.L., Brown, T.L., Esbaugh, A.J., Mager, E.M., Stieglitz, J., Hoenig, R., 2015. Corresponding morphological and molecular indicators of crude oil toxicity to the developing hearts of mahi mahi. Scientific reports 5, 17326.

Ehrlich, K.F., Muszynski, G., 1982. Effects of temperature on interactions of physiological and behavioural capacities of larval California grunion: Adaptations to the planktonic environment. Journal of Experimental Marine Biology and Ecology 60, 223-244.

Esbaugh, A.J., Mager, E.M., Stieglitz, J.D., Hoenig, R., Brown, T.L., French, B.L., Linbo, T.L., Lay, C., Forth, H., Scholz, N.L., Incardona, J.P., Morris, J.M., Benetti, D.D., Grosell, M., 2016. The effects of weathering and chemical dispersion on Deepwater Horizon crude oil toxicity to mahi-mahi (Coryphaena hippurus) early life stages. Science of The Total Environment 543, 644-651.

Farrell, A.P., 2007. Tribute to P. L. Lutz: a message from the heart – why hypoxic bradycardia in fishes? Journal of Experimental Biology 210, 1715.

Forth, H.P., Mitchelmore, C.L., Morris, J.M., Lipton, J., 2017. Characterization of oil and water accommodated fractions used to conduct aquatic toxicity testing in support of the Deepwater Horizon oil spill natural resource damage assessment. Environmental Toxicology and Chemistry 36, 1450-1459.

Frantzen, M., Falk-Petersen, I.B., Nahrgang, J., Smith, T.J., Olsen, G.H., Hangstad, T.A., Camus, L., 2012. Toxicity of crude oil and pyrene to the embryos of beach spawning capelin (Mallotus villosus). Aquatic Toxicology 108, 42-52.

Gemberling, M., Bailey, T.J., Hyde, D.R., Poss, K.D., 2013. The zebrafish as a model for complex tissue regeneration. Trends in Genetics 29, 611-620.

González-Doncel, M., González, L., Fernández-Torija, C., Navas, J.M., Tarazona, J.V., 2008. Toxic effects of an oil spill on fish early life stages may not be exclusively associated to PAHs: studies with Prestige oil and medaka (Oryzias latipes). Aquatic Toxicology 87, 280-288.

Greer, J.B., Pasparakis, C., Stieglitz, J.D., Benetti, D., Grosell, M., Schlenk, D., 2019. Effects of corexit 9500A and Corexit-crude oil mixtures on transcriptomic pathways and developmental toxicity in early life stage mahi-mahi (Coryphaena hippurus). Aquatic Toxicology 212, 233-240.

Grivas, J., Haag, M., Johnson, A., Manalo, T., Roell, J., Das, T.L., Brown, E., Burns, A.R., Lafontant, P.J., 2014. Cardiac repair and regenerative potential in the goldfish (Carassius auratus) heart. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 163, 14-23.

Heard, E., Martienssen, R.A., 2014. Transgenerational epigenetic inheritance: myths and mechanisms. Cell 157, 95-109.

Herrera, C.M., Pozo, M.I., Bazaga, P., 2012. Jack of all nectars, master of most: DNA methylation and the epigenetic basis of niche width in a flower-living yeast. Molecular Ecology 21, 2602-2616.

Hicken, C.E., Linbo, T.L., Baldwin, D.H., Willis, M.L., Myers, M.S., Holland, L., Larsen, M., Stekoll, M.S., Rice, S.D., Collier, T.K., 2011. Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. Proceedings of the National Academy of Sciences 108, 7086-7090.

Ho, D.H., Burggren, W.W., 2012. Parental hypoxic exposure confers offspring hypoxia resistance in zebrafish (Danio rerio). Journal of Experimental Biology, 4208-4216.

Horn, M.A., Trafford, A.W., 2016. Journal of Molecular and Cellular Cardiology Aging and the cardiac collagen matrix : Novel mediators of fi brotic remodelling. Journal of Molecular and Cellular Cardiology 93, 175-185.

Horri, K., Alfonso, S., Cousin, X., Munschy, C., Loizeau, V., Aroua, S., Bégout, M.-l., Ernande, B., 2018. Science of the Total Environment Fish life-history traits are affected after chronic dietary exposure to an environmentally realistic marine mixture of PCBs and PBDEs. Science of the Total Environment 610-611, 531-545.

Hu, L., Xiao, P., Jiang, Y., Dong, M., Chen, Z., Li, H., Hu, Z., Lei, A., Wang, J., 2018. Transgenerational Epigenetic Inheritance Under Environmental Stress by Genome-Wide DNA Methylation Profiling in Cyanobacterium Frontiers in Microbiology 1479.

Huang, L., Gao, D., Zhang, Y., Wang, C., Zuo, Z., 2014. Exposure to low dose benzo [a] pyrene during early life stages causes symptoms similar to cardiac hypertrophy in adult zebrafish. Journal of hazardous materials 276, 377-382.

Hutchinson, T.H., Solbe, J., Kloepper-Sams, P.J., 1998. Analysis of the ecetoc aquatic toxicity (EAT) database III—comparative toxicity of chemical substances to different life stages of aquatic organisms. Chemosphere 36, 129-142.

Inbar-Feigenberg, M., Choufani, S., Butcher, D.T., Roifman, M., Weksberg, R., 2013. Basic concepts of epigenetics. Fertility and sterility 99, 607-615.

Incardona, J.P., 2017. Molecular Mechanisms of Crude Oil Developmental Toxicity in Fish. Archives of Environmental Contamination and Toxicology 73, 19-32.

Incardona, J.P., Carls, M.G., Holland, L., Linbo, T.L., Baldwin, D.H., Myers, M.S., Peck, K.A., Tagal, M., Rice, S.D., Scholz, N.L., 2015. Very low embryonic crude oil exposures cause lasting cardiac defects in salmon and herring. Nature 5, 1-13.

Incardona, J.P., Collier, T.K., Scholz, N.L., 2004. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. 196, 191-205.

Incardona, J.P., Gardner, L.D., Linbo, T.L., Brown, T.L., Esbaugh, A.J., Mager, E.M., Stieglitz, J.D., French, B.L., Labenia, J.S., Laetz, C.A., 2014. Deepwater Horizon crude oil impacts the developing hearts of large predatory pelagic fish. Proceedings of the National Academy of Sciences, 201320950.

Incardona, J.P., Scholz, N.L., 2018a. The 2010 Deepwater Horizon Oil Spill ans Its Environmental Developmental Impacts., in: W.W. Burggren, B. Dubansky (Eds.), Development and Environment, 1 ed. Springer, 235 - 283.

Incardona, J.P., Scholz, N.L., 2018b. Case Study: The 2010 Deepwater Horizon Oil Spill and Its Environmental Developmental Impacts, Development and Environment. Springer, 235-283.

Incardona, J.P., Vines, C.a., Anulacion, B.F., Baldwin, D.H., Day, H.L., French, B.L., Labenia, J.S., Linbo, T.L., Myers, M.S., Olson, O.P., Sloan, C.a., Sol, S., Griffin, F.J., Menard, K., Morgan, S.G., West, J.E., Collier, T.K., Ylitalo, G.M., Cherr, G.N., Scholz, N.L., 2012. PNAS Plus: Unexpectedly high mortality in Pacific herring embryos exposed to the 2007 Cosco Busan oil spill in San Francisco Bay. Proceedings of the National Academy of Sciences 109, E51-E58.

Jablonka, E., Lamb, M.J., 2015. The inheritance of acquired epigenetic variations. International Journal of Epidemiology 44, 1094-1103.

Jablonka, E., Raz, G., 2009. Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. The Quarterly Review of Biology 84, 131-176.

Jaspers, R.T., Testerink, J., Della Gaspera, B., Chanoine, C., Bagowski, C.P., van der Laarse, W.J., 2014. Increased oxidative metabolism and myoglobin expression in zebrafish muscle during chronic hypoxia. Biology Open 3, 718 LP - 727.

Johansen, J.L., Allan, B.J.M., Rummer, J.L., Esbaugh, A.J., 2017. Oil exposure disrupts early life-history stages of coral reef fishes via behavioural impairments. Nature Ecology and Evolution 1, 1146-1152.

Khursighara, A.J., Perrichon, P., Bautista, N.M., Burggren, W.W., Esbaugh, A.J., 2016. Cardiac function and survival are affected by crude oil in larval red drum, Sciaenops ocellatus. Science of the Total Environment 579, 797-804.

Kikuchi, K., 2014. ScienceDirect Advances in understanding the mechanism of zebrafish heart regeneration. 13, 542-555.

Kimmel, C.B.C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Developmental … 10, 253-310.

Klironomos, F.D., Berg, J., Collins, S., 2013. How epigenetic mutations can affect genetic evolution: model and mechanism. Bioessays 35, 571-578.

Laubach, Z.M., Perng, W., Dolinoy, D.C., Faulk, C.D., Holekamp, K.E., Getty, T., 2018. Epigenetics and the maintenance of developmental plasticity: extending the signalling theory framework. Biological Reviews.

Lema, S.C., Schultz, I.R., Scholz, N.L., Incardona, J.P., Swanson, P., 2007. Neural defects and cardiac arrhythmia in fish larvae following embryonic exposure to 2 , 2 , 4 , 4 -tetrabromodiphenyl ether (PBDE 47). 82, 296-307.

Lester, B.M., Conradt, E., Marsit, C., 2016. Introduction to the special section on epigenetics. Child Development 87, 29-37.

Lombó, M., Fernández-Díez, C., González-Rojo, S., Navarro, C., Robles, V., Herráez, M.P., 2015. Transgenerational inheritance of heart disorders caused by paternal bisphenol A exposure. Environmental pollution 206, 667-678.

Mager, E., Pasparakis, C., Schlenker, L.S., Yao, Z., Bodinier, C., Stieglitz, J.D., Hoenig, R., Morris, J.M., Benetti, D.D., Grosell, M., 2017. Assessment of early life stage mahi-mahi windows of sensitivity during acute exposures to Deepwater Horizon crude oil. Environmental Toxicology and Chemistry 36, 1887-1895.

Mager, E.M., Esbaugh, A.J., Stieglitz, J.D., Hoenig, R., Bodinier, C., Incardona, J.P., Scholz, N.L., Benetti, D.D., Grosell, M., 2014. Acute embryonic or juvenile exposure to deepwater horizon crude oil impairs the swimming performance of mahi-mahi (Coryphaena hippurus). Environmental Science and Technology 48, 7053-7061.

Mager, E.M., Grosell, M., 2011. Effects of acute and chronic waterborne lead exposure on the swimming performance and aerobic scope of fathead minnows (Pimephales promelas). Comparative Biochemistry and Physiology - C Toxicology and Pharmacology 154, 7-13.

Manjrekar, J., 2017. Epigenetic inheritance , prions and evolution. Journal of Genetics 96, 445-456.

Marro, J., Pfefferli, C., Charles, A.-s.D.P., Bise, T., Ja, A., 2016. Collagen XII Contributes to Epicardial and Connective Tissues in the Zebrafish Heart during Ontogenesis and Regeneration. 1-23.

McKim, J.M., 1977. Evaluation of Tests with Early Life Stages of Fish for Predicting Long-Term Toxicity. Journal of the Fisheries Research Board of Canada 34, 1148-1154.

Meyer, J., Di Giulio, R., 2002. Patterns of heritability of decreased EROD activity and resistance to PCB 126-induced teratogenesis in laboratory-reared offspring of killifish (Fundulus heteroclitus) from a creosote-contaminated site in the Elizabeth River, VA, USA. Marine Environmental Research 54, 621-626.

Meyer, J.N., Di Giulio, R.T., 2003. Heritable Adaptation and Fitness Costs in Killifish (Fundulus Heteroclitus) Inhabiting a Polluted Estuary. Ecological Applications 13, 490-503.

Meyer, J.N., Nacci, D.E., Giulio, R.T.D., 2002. Cytochrome P4501A (CYP1A) in Killifish (Fundulus heteroclitus): Heritability of Altered Expression and Relationship to Survival in Contaminated Sediments. Toxicological Sciences 68, 69-81.

Milash, B., Gao, J., Stevenson, T.J., Son, J.-H., Dahl, T., Bonkowsky, J.L., 2016. Temporal Dysynchrony in brain connectivity gene expression following hypoxia. BMC genomics 17, 334.

Mohammed, A., 2013. Why are early life stages of aquatic organisms more sensitive to toxicants than adults?, New insights into toxicity and drug testing. InTech.

Moosavi, A., Ardekani, A.M., 2016. Role of epigenetics in biology and human diseases. Iranian Biomedical Journal 20, 246-258.

Motta, S.S., Cluzel, P., Aldana, M., 2015. Adaptive Resistance in Bacteria Requires Epigenetic Inheritance, Genetic Noise, and Cost of Efflux Pumps. PLOS ONE 10, e0118464.

Nacci, D.E., Champlin, D., Jayaraman, S., 2010. Adaptation of the Estuarine Fish Fundulus heteroclitus (Atlantic Killifish) to Polychlorinated Biphenyls (PCBs). Estuaries and Coasts 33, 853-864.

Nelson, D., Heuer, R.M., Cox, G.K., Stieglitz, J.D., Hoenig, R., Mager, E.M., Benetti, D.D., Grosell, M., Crossley, D.A., 2016. Effects of crude oil on in situ cardiac function in young adult mahi???mahi (Coryphaena hippurus). Aquatic Toxicology 180, 274-281.

Nelson, D., Stieglitz, J.D., Cox, G.K., Heuer, R.M., Benetti, D.D., Grosell, M., Crossley, D.A., 2017. Cardio-respiratory function during exercise in the cobia, Rachycentron canadum: The impact of crude oil exposure. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 201, 58-65.

Nie, M., Blankenship, A.L., Giesy, J.P., 2001. Interactions between aryl hydrocarbon receptor (AhR) and hypoxia signaling pathways. Environmental Toxicology and Pharmacology 10, 17-27.

Nye, J.A., Davis, D.D., Miller, T.J., 2007. The effect of maternal exposure to contaminated sediment on the growth and condition of larval Fundulus heteroclitus. 82, 242-250.

Ownby David, R., Newman Michael, C., Mulvey, M., Vogelbein Wolfgang, K., Unger Michael, A., Arzayus, L.F., 2009. Fish (Fundulus heteroclitus) populations with different exposure histories differ in tolerance of creosote-contaminated sediments. Environmental Toxicology and Chemistry 21, 1897-1902.

Pasparakis, C., Esbaugh, A.J., Burggren, W., Grosell, M., 2019. Impacts of deepwater horizon oil on fish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 224, 108558.

Pasparakis, C., Mager, E.M., Stieglitz, J.D., Benetti, D., Grosell, M., 2016. Effects of Deepwater Horizon crude oil exposure, temperature and developmental stage on oxygen consumption of embryonic and larval mahi-mahi (Coryphaena hippurus). Aquatic Toxicology 181, 113-123.

Pasparakis, C., Sweet, L.E., Stieglitz, J.D., Benetti, D., Casente, C.T., Roberts, A.P., Grosell, M., 2017. Combined effects of oil exposure, temperature and ultraviolet radiation on buoyancy and oxygen consumption of embryonic mahi-mahi, Coryphaena hippurus. Aquatic Toxicology 191, 113-121.

Pelster, B., Grillitsch, S., Schwerte, T., 2005. NO as a mediator during the early development of the cardiovascular system in the zebrafish B. 142, 215-220.

Perrichon, P., Akcha, F., Le Menach, K., Goubeau, M., Budzinski, H., Cousin, X., Bustamante, P., 2015. Parental trophic exposure to three aromatic fractions of polycyclic aromatic hydrocarbons in the zebrafish: Consequences for the offspring. Science of The Total Environment 524-525, 52-62.

Perrichon, P., Le Menach, K., Akcha, F., Cachot, J.r.m., Budzinski, H.l.n., Bustamante, P., 2016. Toxicity assessment of water-accommodated fractions from two different oils using a zebrafish (Danio rerio) embryo-larval bioassay with a multilevel approach. Science of the Total Environment 568, 952-966.

Perry, S.F., Desforges, P.R., 2006. Does bradycardia or hypertension enhance gas transfer in rainbow trout (Oncorhynchus mykiss)? Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 144, 163-172.

Pitt, J.A., Kozal, J.S., Jayasundara, N., Massarsky, A., Trevisan, R., Geitner, N., Wiesner, M., Levin, E.D., Di Giulio, R.T., 2018. Uptake, tissue distribution, and toxicity of polystyrene nanoparticles in developing zebrafish (Danio rerio). Aquatic Toxicology 194, 185-194.

Réalis-Doyelle, E., Pasquet, A., De Charleroy, D., Fontaine, P., Teletchea, F., 2016. Strong Effects of Temperature on the Early Life Stages of a Cold Stenothermal Fish Species, Brown Trout (Salmo trutta L.). PLOS ONE 11, e0155487.

Reddam, A., Mager, E.M., Grosell, M., McDonald, M.D., 2017. The impact of acute PAH exposure on the toadfish glucocorticoid stress response. Aquatic Toxicology 192, 89-96.

Schrey, W.A., Richards, L.C., 2012. Within-genotype epigenetic variation enables broad niche width in a flower living yeast. Molecular Ecology 21, 2559-2561.

Schwerte, T., Prem, C., Mairösl, A., Pelster, B., 2006. Development of the sympatho-vagal balance in the cardiovascular system in zebrafish (Danio rerio) characterized by power spectrum and classical signal analysis. 1093-1100.

Seemann, F., Jeong, C.-B., Zhang, G., Wan, M.T., Guo, B., Peterson, D.R., Lee, J.-S., Au, D.W.-T., 2017. Ancestral benzo[a]pyrene exposure affects bone integrity in F3 adult fish (Oryzias latipes). Aquatic Toxicology 183, 127-134.

Seemann, F., Peterson, D.R., Witten, P.E., Guo, B.-S., Shanthanagouda, A.H., Ye, R.R., Zhang, G., Au, D.W.T., 2015. Insight into the transgenerational effect of benzo[a]pyrene on bone formation in a teleost fish (Oryzias latipes). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 178, 60-67.

Sheehan, D.C., Hrapchak, B.B., 1980. Theory and practice of histotechnology. Cv Mosby.

Siefert, R.E., Spoor, W.A., Syrett, R.F., 1973. Effects of Reduced Oxygen Concentrations on Northern Pike (Esox lucius) Embryos and Larvae. Journal of the Fisheries Research Board of Canada 30, 849-852.

Skinner, M.K., 2015. Environmental Epigenetics and a Unified Theory of the Molecular Aspects of Evolution: A Neo-Lamarckian Concept that Facilitates Neo-Darwinian Evolution. Genome Biology and Evolution 7, 1296-1302.

Sørhus, E., Incardona, J.P., Furmanek, T., Goetz, G.W., Scholz, N.L., Meier, S., Edvardsen, R.B., Jentoft, S., 2017. Novel adverse outcome pathways revealed by chemical genetics in a developing marine fish. eLife 6, e20707.

Spence, R., Gerlach, G., Lawrence, C., Smith, C., 2008. The behaviour and ecology of the zebrafish, Danio rerio. Biological Reviews 83, 13-34.

Stieglitz, J.D., Mager, E.M., Hoenig, R.H., Benetti, D.D., Grosell, M., 2016. Impacts of Deepwater Horizon crude oil exposure on adult mahi-mahi (Coryphaena hippurus) swim performance. Environmental Toxicology and Chemistry 35, 2613-2622.

Thorson, J.L.M., Smithson, M., Beck, D., Sadler-Riggleman, I., Nilsson, E., Dybdahl, M., Skinner, M.K., 2017. Epigenetics and adaptive phenotypic variation between habitats in an asexual snail. Scientific Reports 7, 14139.

Tierney, K.B., Kennedy, C.J., Gobas, F., Gledhill, M., Sekela, M., 2013. Organic contaminants and fish, Fish Physiology. Elsevier, 1-52.

Timme-Laragy, A.R., Meyer, J.N., Waterland, R.A., Di Giulio, R.T., 2005. Analysis of CpG methylation in the killifish CYP1A promoter. Comparative biochemistry and physiology. Toxicology & pharmacology : CBP 141, 406-411.

Vignet, C., Menach, K.L., Mazurais, D., Lucas, J., Perrichon, P., Bihanic, F.L., Devier, M.-h., Lyphout, L., Frère, L., 2014. Chronic dietary exposure to pyrolytic and petrogenic mixtures of PAHs causes physiological disruption in zebrafish - part I : Survival and growth.

Vogt, G., 2017. Facilitation of environmental adaptation and evolution by epigenetic phenotype variation: insights from clonal, invasive, polyploid, and domesticated animals. Environmental Epigenetics 3, dvx002-dvx002.

Westerfield, M., 2007. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 5th Edition. University of Oregon Press, Eugene (Book).

Williams, J., 2000. The coefficient of condition of fish. The manual of fisheries survey methods II: with periodic updates. Michigan department of natural resources, Fisheries special report 25.

Wilson-Leedy, J.G., Ingermann, R.L., 2007. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 67, 661-672.

Woltering, D.M., 1984. The growth response in fish chronic and early life stage toxicity tests: A critical review. Aquatic Toxicology 5, 1-21.

Xu, E.G., Khursigara, A.J., Magnuson, J., Hazard, E.S., Hardiman, G., Esbaugh, A.J., Roberts, A.P., Schlenk, D., 2017a. Larval Red Drum (Sciaenops ocellatus) Sublethal Exposure to Weathered Deepwater Horizon Crude Oil: Developmental and Transcriptomic Consequences. Environmental Science & Technology 51, 10162-10172.

Xu, E.G., Mager, E.M., Grosell, M., Stieglitz, J.D., Hazard, E.S., Hardiman, G., Schlenk, D., 2017b. Developmental transcriptomic analyses for mechanistic insights into critical pathways involved in embryogenesis of pelagic mahi-mahi (Coryphaena hippurus). PLOS ONE 12, e0180454.

Zhou, R., Lu, G., Yan, Z., Jiang, R., Shen, J., Bao, X., 2019. Parental transfer of ethylhexyl methoxy cinnamate and induced biochemical responses in zebra fi sh. Aquat Toxicol 206, 24-32.

Tables

Table 1. Fecundity variables resulting from HEWAF exposure in adult male and female zebrafish. Different superscript letters indicate differences among dietary treatment groups.

A) *Fecundity variables*	Treatment				p value
	Control	10%HEWAF	50%HEWAF	100%HEWAF	
"n" number	15	15	15	15	$\alpha = 0.05$
Spawned (#Yes/#No)	15/0 ^{A}	15/0 ^{A}	14/1 ^{A}	7/8 ^{B}	0.0001
Total egg #	5352	4130	2366	2090	NA
Average egg # / female	356.8 ± 51.7 ^{A}	275.3 ± 28.8 ^{AB}	157.7 ± 23.9 ^{BC}	139.3 ± 40.7 ^{C}	0.002
% of fertilized eggs	75.8 ± 5.4 ^{A}	38 ± 4.6 ^{B}	61 ± 5.9 ^{B}	58.9. ± 8.1 ^{B}	0.001
% of non-fertilized eggs	24.2 ± 5.4 ^{A}	62 ± 4.6 ^{B}	43.3 ± 7 ^{AB}	41.1 ± 8.1 ^{AB}	0.001
% of non-viable fertilized eggs	5.9 ± 2.7 ^{A}	38.1 ± 5 ^{B}	26.1 ± 7.1 ^{AB}	14.6 ± 6.3 ^{AB}	0.001

Table 2. Egg variables **A)**, and sperm quality variables **B)** in female and male zebrafish exposed to varying HEWAF concentrations. Different superscript letters indicate differences among dietary treatment groups.

A) *Egg variables*	Treatment				p value
	Control	10%HEWAF	50%HEWAF	100%HEWAF	
"n" number	25	25	25	25	α = 0.05
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

B) *Sperm quality variables*	Treatment				p value
	Control	10%HEWAF	50%HEWAF	100%HEWAF	
"n" number	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 (VCL)	55.7 ± 5.7	57.3 ± 6.7	58.8. ± 9	78 ± 10.4	0.202
Velocity average path (VAP)	41.7 ± 2.3	37.1 ± 4.6	46.3 ± 3.8	48.3 ± 3.6	0.165
Velocity straigth line (VSL)	27.1 ± 2.1	23.9 ± 5.8	35.7 ± 1.3	25.8 ± 2.9	0.104
Linearity (LIN)	66 ± 6.4	60.8 ± 13.5	74.9 ± 6.5	67 ± 12.3	0.145
Count / 0.006 mm^2	18 ± 1.4 ^{A}	10.8 ± 1.7 ^{B}	9.4 ± 2.5 ^{B}	9.3 ± 3 ^{B}	0.039

Table 3. Fecundity variables resulting from HEWAF exposure in adult male and female zebrafish. Different superscript letters indicate differences among dietary treatment groups. * and ** indicate that the value of the adjusted residuals from the Chi-square analysis were equal to (or beyond) 2 or -2 , which deviate them from the H_0 (equal proportions) respectively (Agresti and Kateri, 2011).

Exposure condition		% of individuals exhibiting malformities				SGR
Parental	F_1 Offspring	Cardiac edema	Yolk edema	Head deformities	Tail deformities	% body length/day
		n = 15	n = 15	n = 15	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 Chi square / 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 Chi square / 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 Chi square / 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 Chi square / P value		10.2 / 0.017	4.845 / 0.184	19.615 / 0.0001	12.274 / 0.007	

Figures

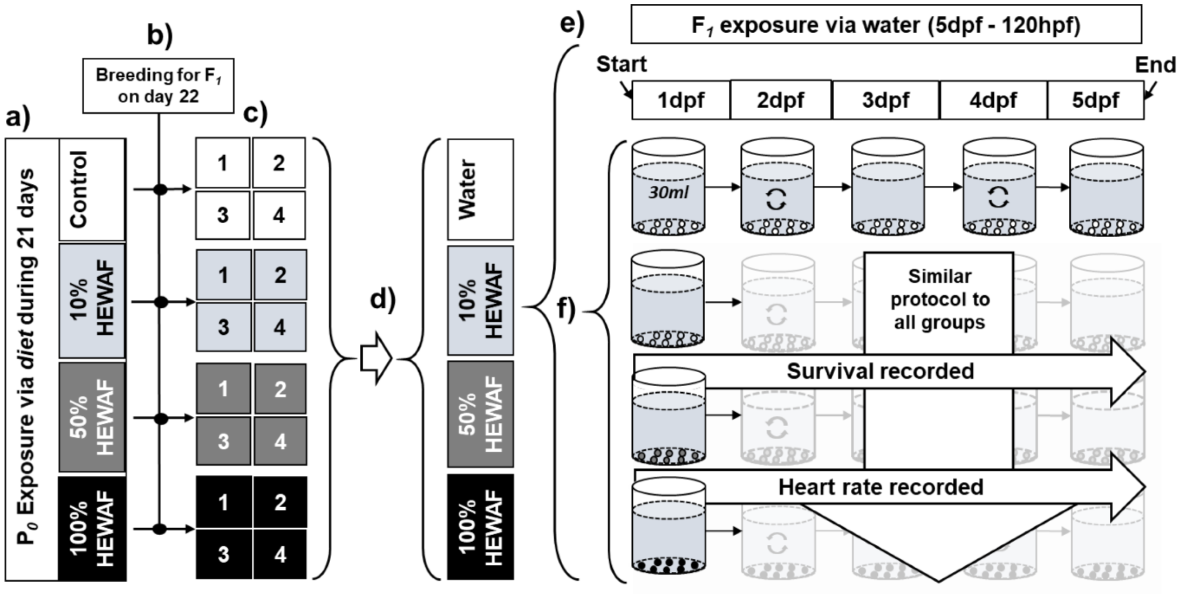

Fig. 1. Experimental protocol. **a)** A parental population of adult zebrafish was divided into 4 exposure groups and exposed via diet to water or any of the HEWAF diets for 21 days. **b)** Offspring (F_1) were obtained from breeding within each one of the parental groups on day 22. **c)** The F_1 larvae from each group was subsequently divided into 4 subgroups, 1 to 4. **d)** One subgroup from each F_1 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. **e)** Exposure to 10% HEWAF began at ~ 3 hours post fertilization and ended at 5dpf. **f)** The F_1 from the four different parental groups were placed into 50ml beakers filled with 30ml of water or one of the three HEWAF solutions. To maintain levels of exposure, the solution was changed on day 2 and 4. Survival and heart rate recordings on all populations were performed throughout the 5 days of HEWAF exposure.

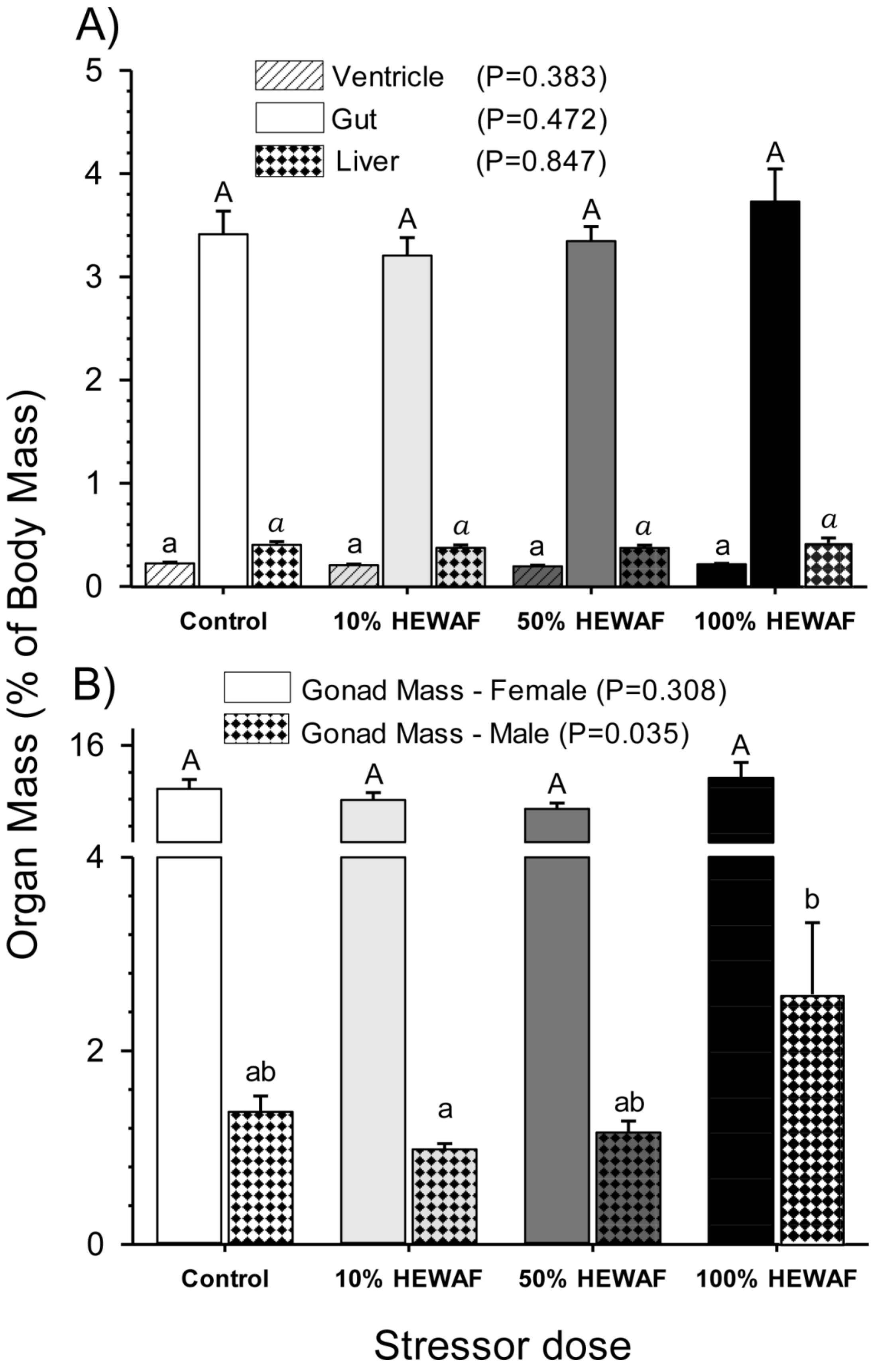
A)
Ventricle (P=0.383)
Gut (P=0.472)
Liver (P=0.847)
5
4
3
2
1
0
a
A
a
Control
10% HEWAF
50% HEWAF
100% HEWAF
B)
Gonad Mass - Female (P=0.308)
Gonad Mass - Male (P=0.035)
16
4
2
0
A
ab
a
ab
b
Organ Mass (% of Body Mass)
Stressor dose

Fig. 2. Adult organ mass as percentage of whole body mass, as influenced by crude oil exposure. A) Comparison of: ventricle, gut and liver mass between treatments (n=27-30/bar). **B)** Female (n=15–18/bar) and male (n=11-12/bar) gonadal mass. Data are presented as mean ± SEM. Significance level was considered with P value < 0.05. Different letters indicate statistical significant differences between groups.

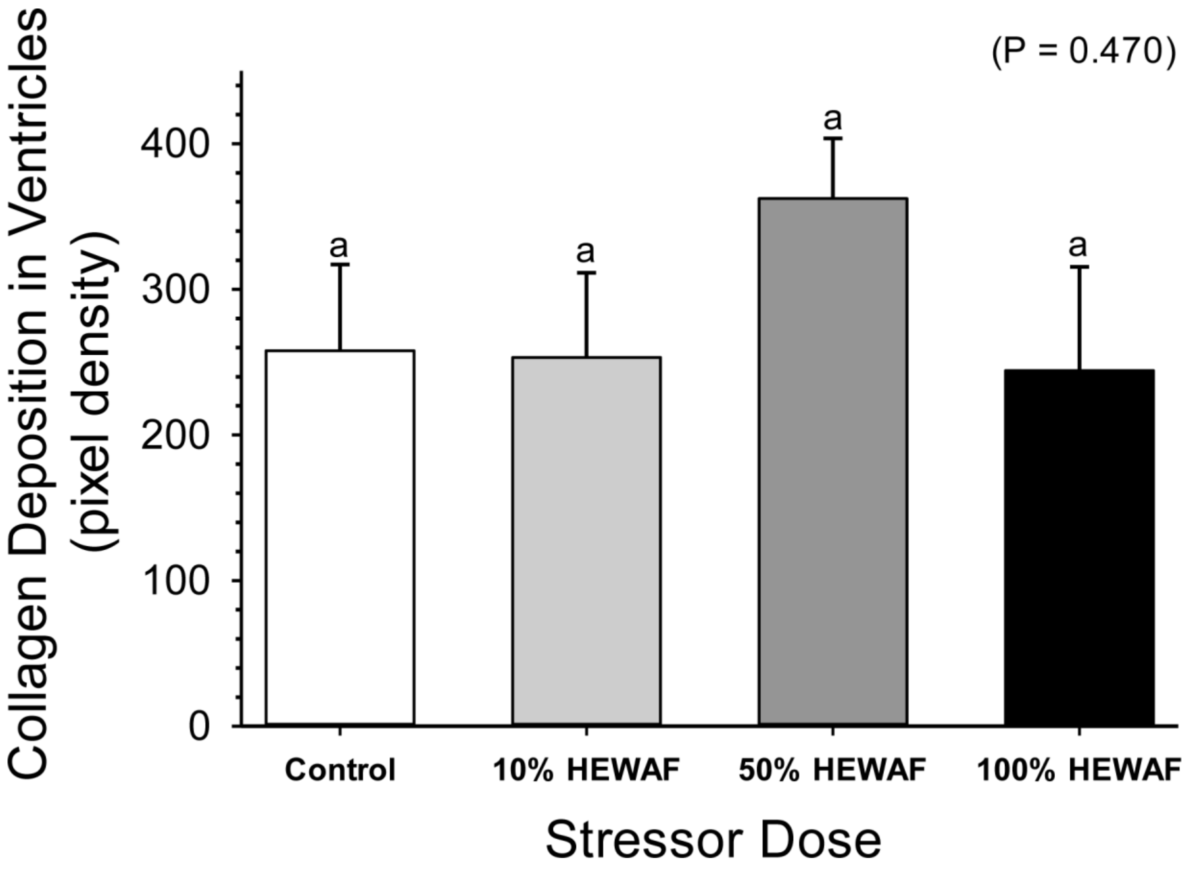

Fig. 3. Collagen in adult ventricles, as influenced by crude oil exposure. Collagen area is expressed as pixel density, in the images of the ventricles from adult zebrafish exposed to different levels of HEWAF. There were no statistical significant differences between treatments (mean ± SEM, P=0.470). n=6-7 per group.

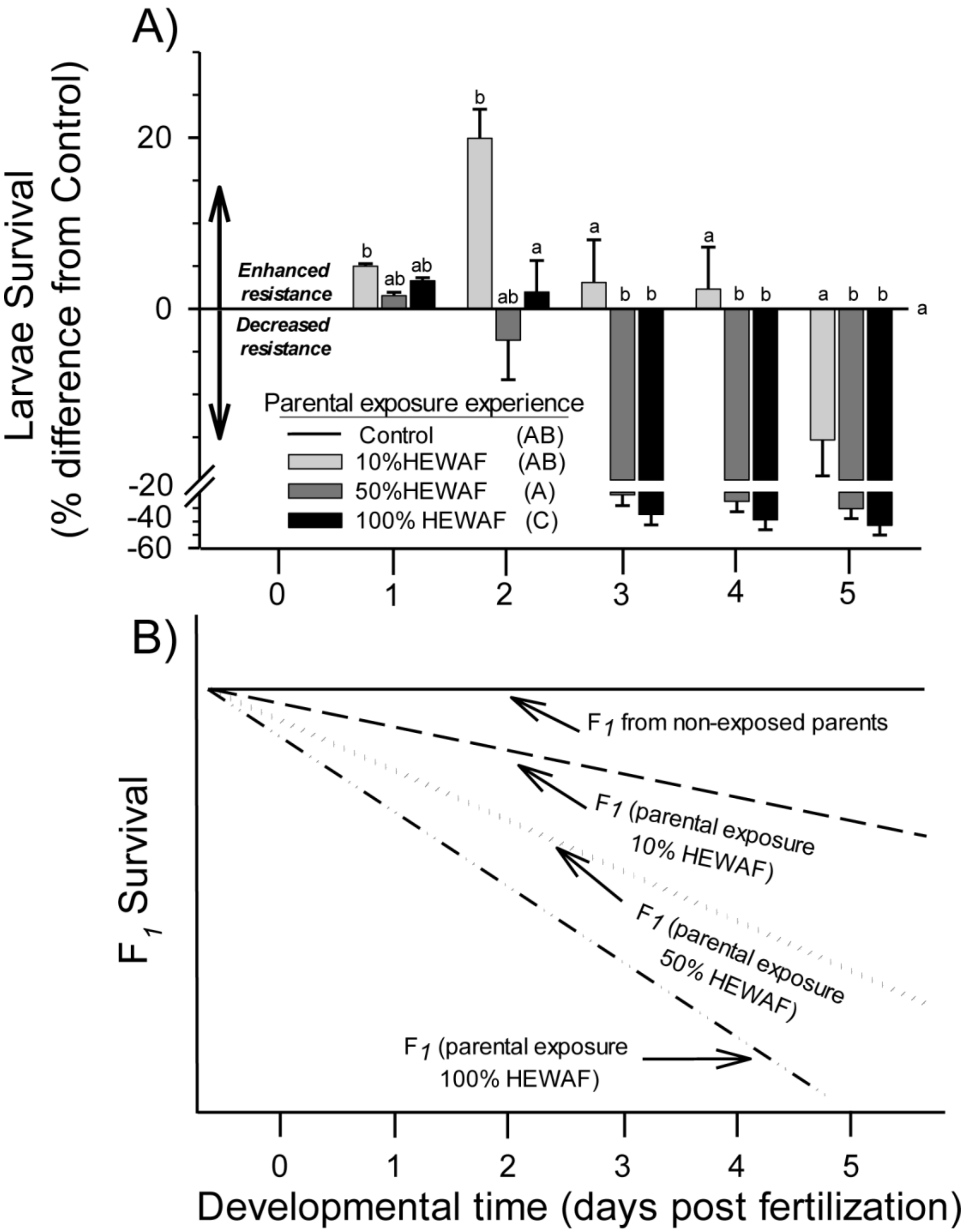

Fig. 4. Effect of parental HEWAF exposure on larval survival. A) Survival of F1 larvae raised in clean water. Presented are the differences in survival % between F_1 obtained from control parents (zero-line), and F_1 obtained from treated parental groups (bars), at

specific developmental time (dpf). Bars above or below the zero-line are interpreted as enhanced or decreased resistance, respectively. Different upper case letters by each parental treatment in the legend indicate significant ($P<0.001$) differences occurred between populations. Different lower case letters above the bars indicate difference between groups at specific days. “a” was assigned to the control group for all days, and is showed at the end of the zero-line. B) Scheamtic representation of survival patterns of F_1 from exposed parents raised in clean water, derived from Panel A. Each bar in panel A represents mean and SEM of the three replicates. The backwards arrows are just indicating which label belongs to each treatment one of the conceptual trends.

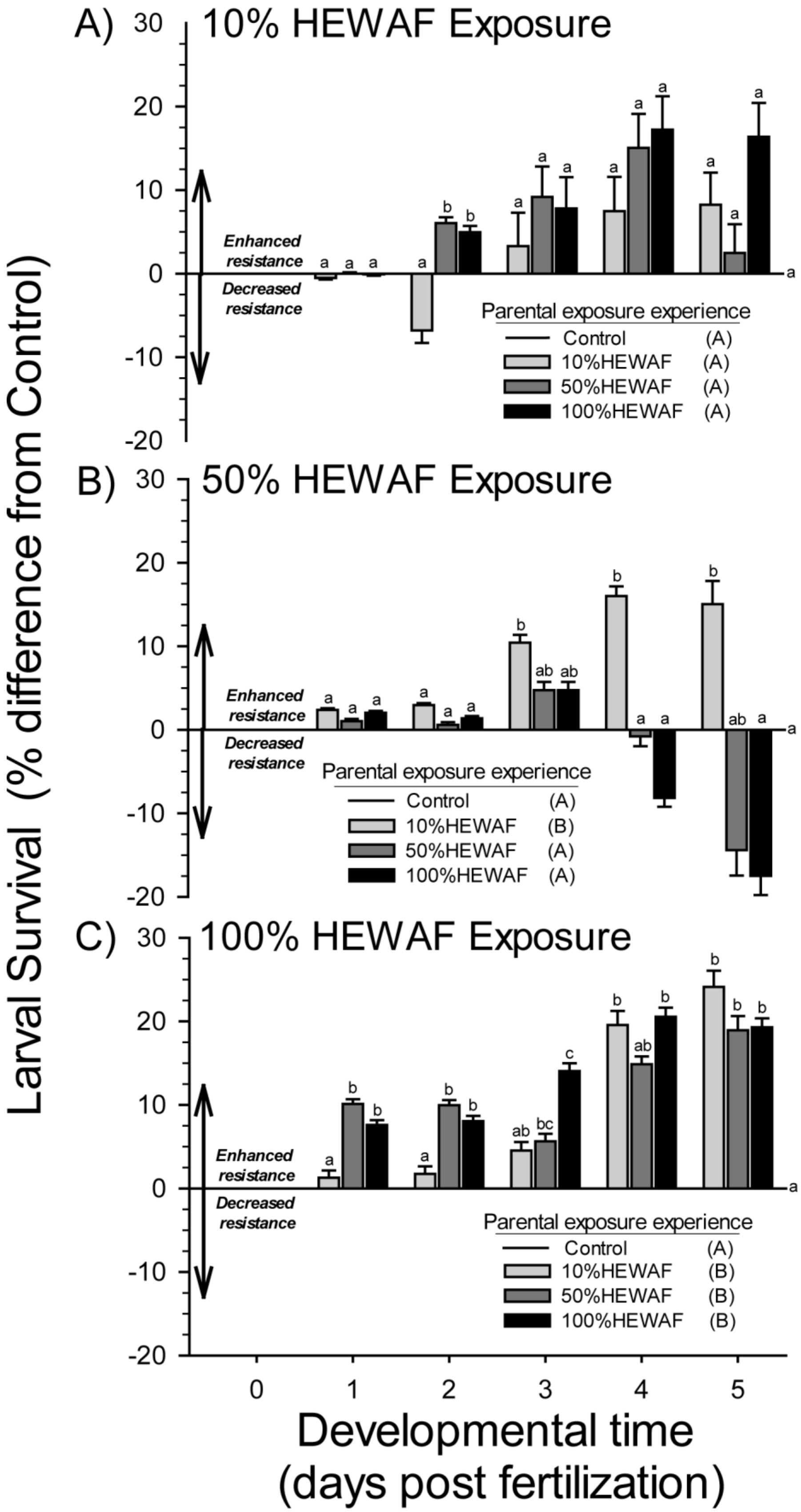
A) 10% HEWAF Exposure
B) 50% HEWAF Exposure
C) 100% HEWAF Exposure
Larval Survival (% difference from Control)
30
20
10
0
-10
-20
Enhanced resistance
Decreased resistance
Parental exposure experience
Control (A)
10%HEWAF (A)
50%HEWAF (A)
100%HEWAF (A)
Control (A)
10%HEWAF (B)
50%HEWAF (A)
100%HEWAF (A)
Control (A)
10%HEWAF (B)
50%HEWAF (B)
100%HEWAF (B)
a
b
ab
bc
c
0
1
2
3
4
5
Developmental time (days post fertilization)

Fig.5. Synergistic and antagonistic effects of parental and larval HEWAF exposure on larval survivorship. A) F_1 larval exposure to 10% HEWAF. B) F_1 larval exposure to 50% HEWAF. C) F_1 larval exposure to 100% HEWAF. Data are presented as the difference in survival % between F_1 obtained from control parents (zero-line), and F_1 obtained from treated parental groups (bars), at specific developmental time (dpf). Bars above or below the zero-line are interpreted as enhanced or decreased resistance respectively. Different upper case letters by each parental treatment in the legend indicate significant ($P<0.001$) differences occurred between populations. Different lower case letters above the bars indicate difference between groups at specific days, “a” was assigned to the control group for all days, and is showed at the end of the zero-line. See Results for additional explanation. Each bar represents mean and SEM of the three replicates.

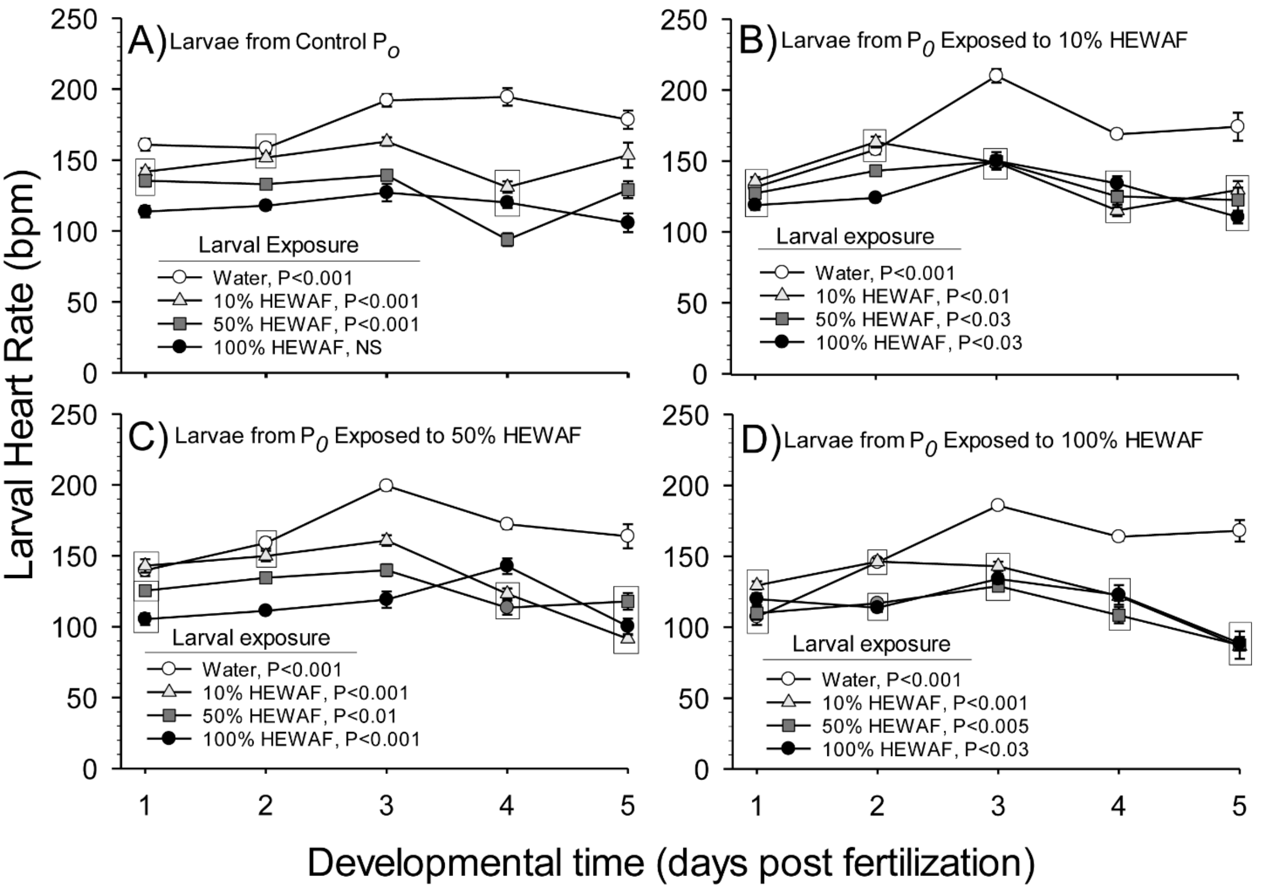

Fig. 6. Heart rate in 1 to 5 dpf F_1 zebrafish larvae as a function of P_0 parental crude oil exposure. **A)** Larvae from control parents, **B)** Larvae from 10%HEWAF exposed parents, **C)** Larvae from 50%HEWAF exposed parents and **D)** Larvae from 100%HEWAF exposed parents. Larvae raised in Clean water, 10%HEWAF, 50%HEWAF or 100%HEWAF are indicated with white circles, gray triangles, dark-gray squares, and black circles, respectively. Data are expressed as means ± 1 SEM. Means for any given developmental day that are grouped within boxes are not significantly different (P>0.05). P-values beside the legend refer to differences across developmental time for each treatment. n= 8-74 per data point.

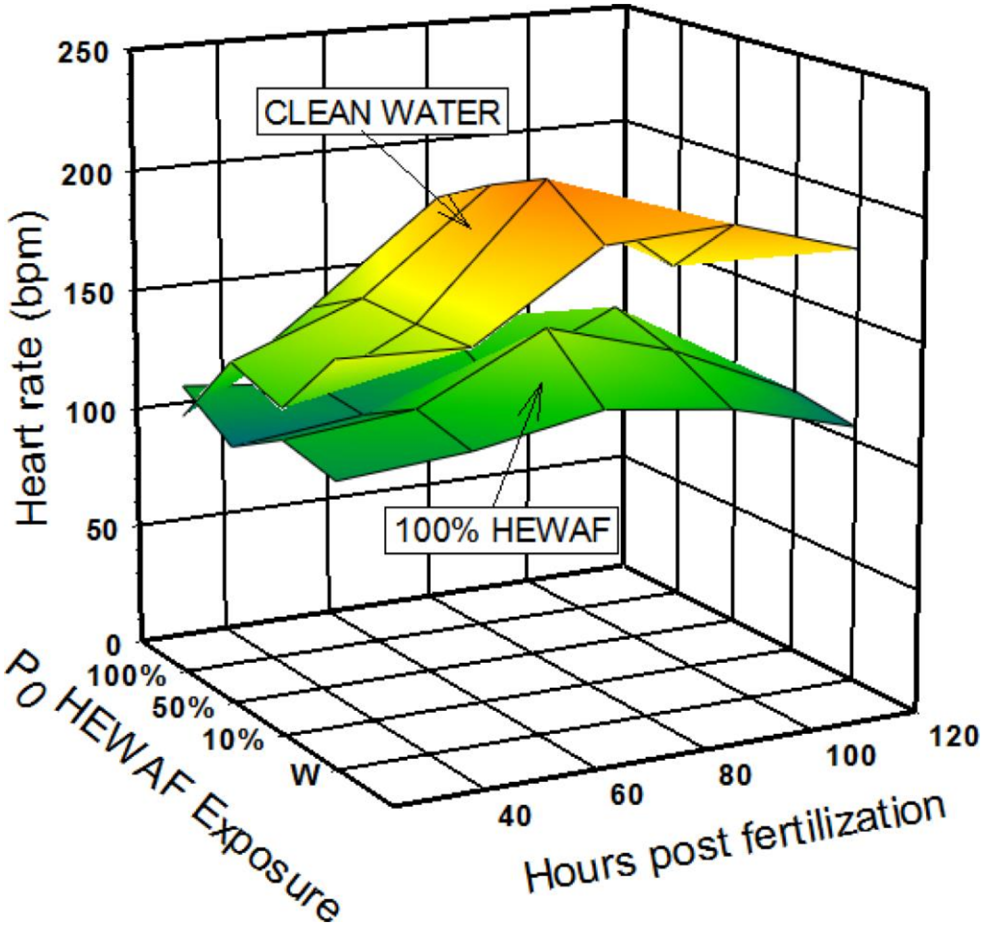

Fig. 7. Comparison of heart rate between offspring exposed to clean water (top plane) and offspring exposed to 100% HEWAF (bottom plane). Surfaces for 10% and 50% HEWAF exposures are intermediate and have been omitted for clarity.

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.

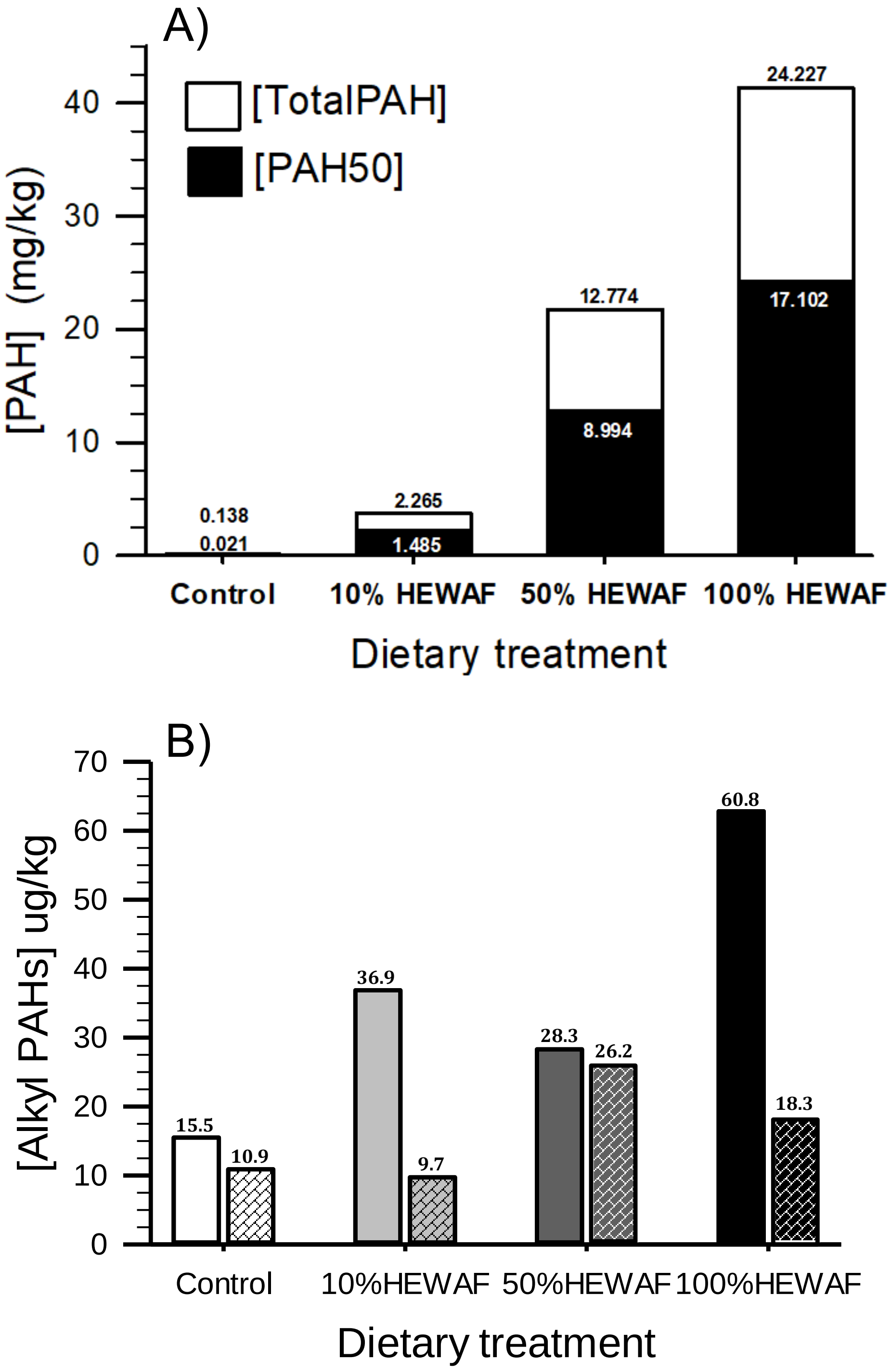

Fig. S2. Male gonadal sections. A) Control, B) 10%HEWAF, C) 50%HEWAF and D) 100%HEWAF. L= lumina, S= spermatogonia, SP= spermatocysts

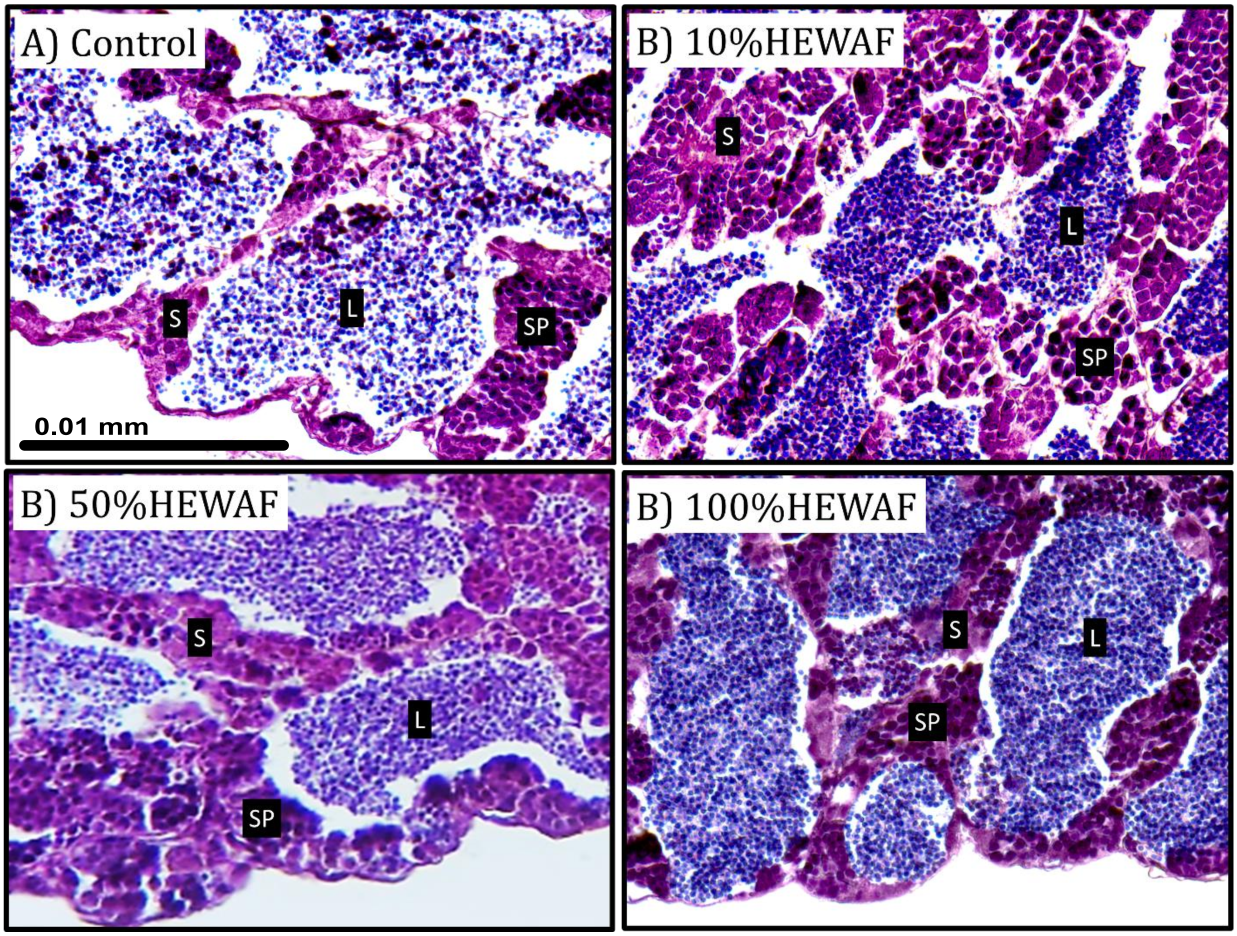

Fig. S3. Female gonadal sections. A) Control, B) 10%HEWAF, C) 50%HEWAF and D) 100%HEWAF. PO = Previtellogenic oocytes, VO = vitellogenic oocytes.

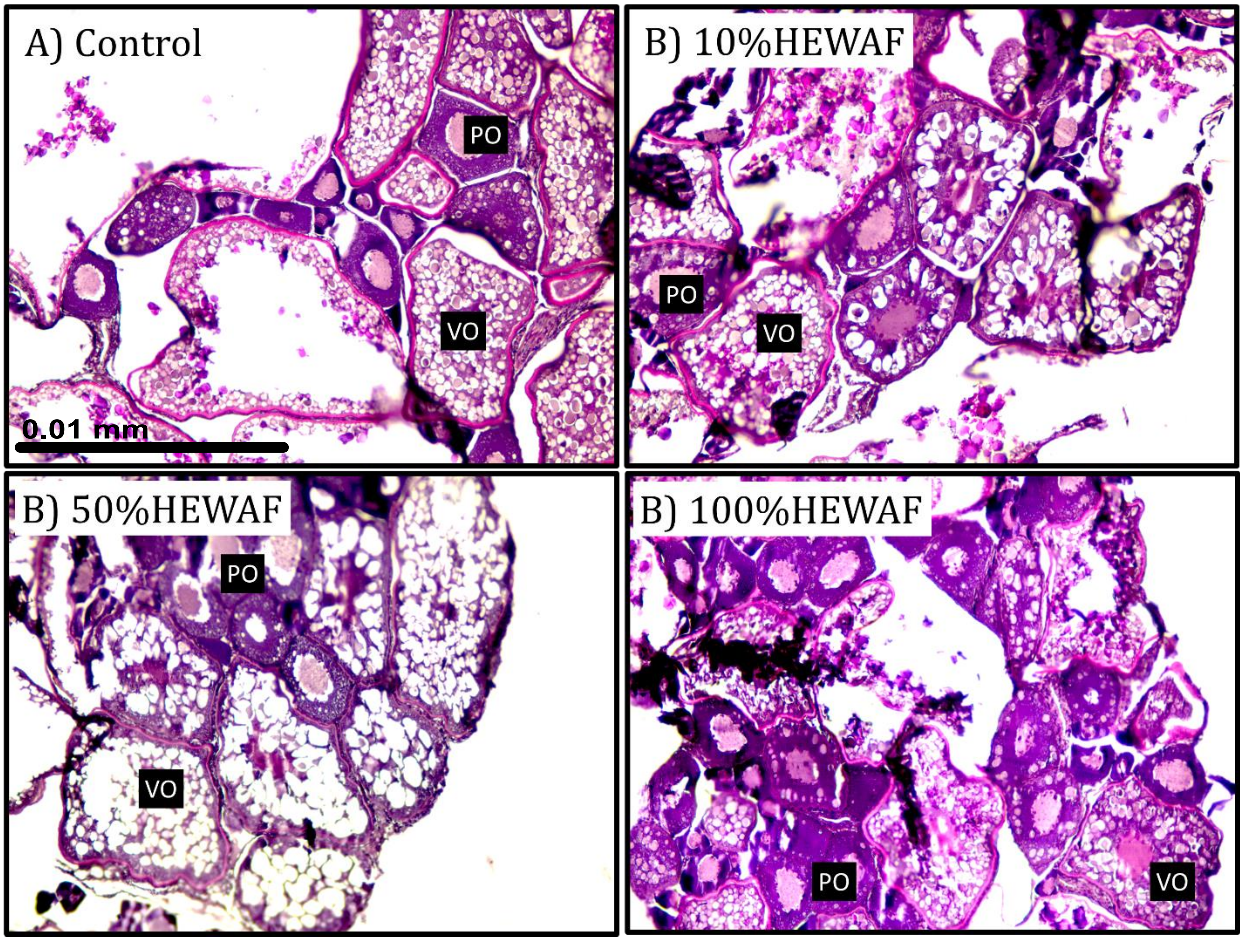

Fig. S4. Masson's trichrome staining technique in ventricular tissue. Positive control stains, **A)** gill tissue and **B)** bulbus arteriousus tissue. C) Control, D) 10%HEWAF, E) 50%HEWAF and F) 100%HEWAF groups, respectively.

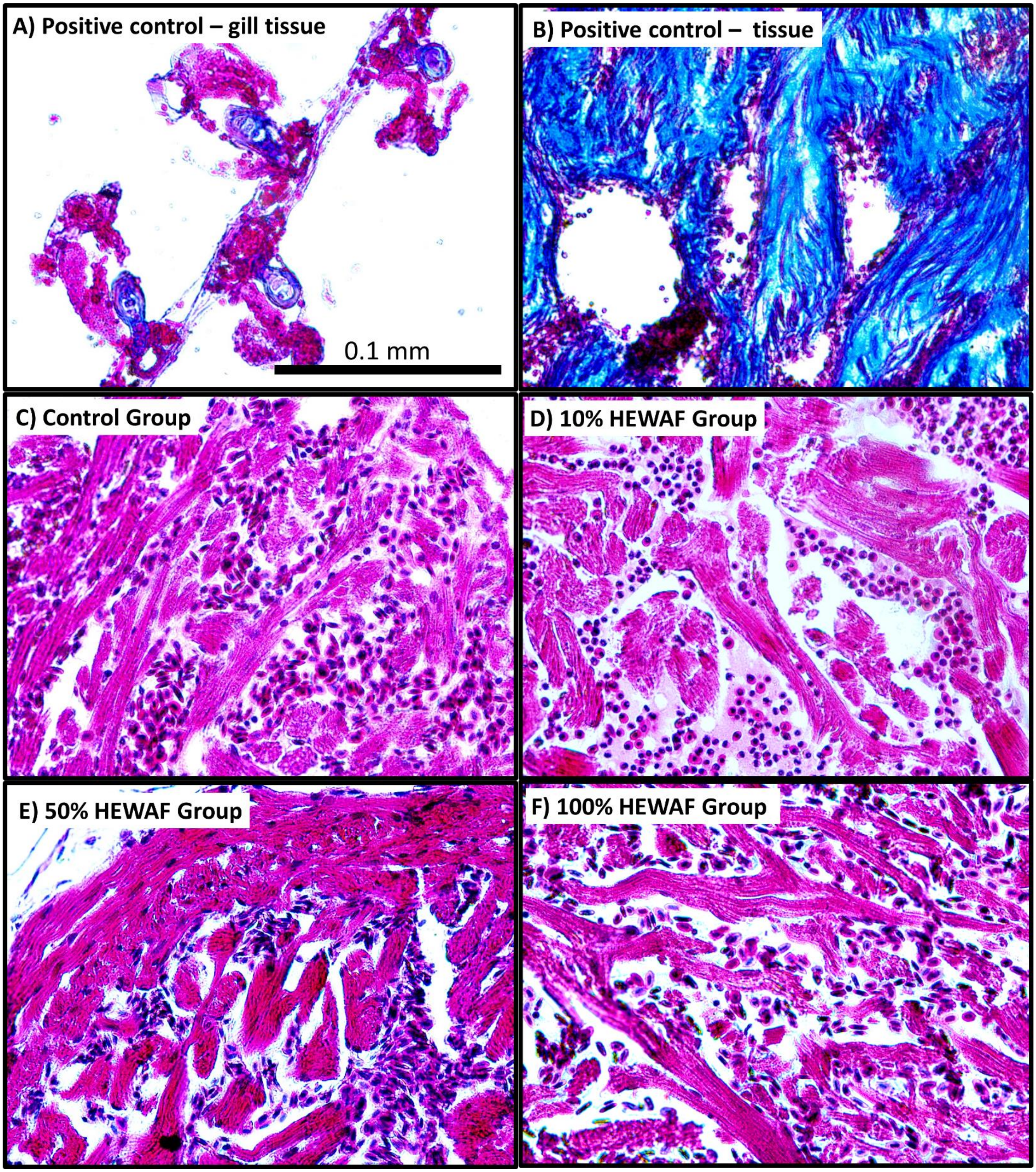

Table. S1. List of components and nominal concentrations (ug/Kg and mg/kg) for each diet treatment. The sum of all the components listed below was considered the “Total PAH concentration”. The components highlighted with gray color were considered for the 50 PAHs most frequently measured PAHs (Dubansky 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

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

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

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