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RESEARCH ARTICLE

Acute exposure of early-life stage zebrafish (*Danio rerio*) to *Deepwater Horizon* crude oil impairs glomerular filtration and renal fluid clearance capacity

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Abstract

The pronephros (early-stage kidney) is an important osmoregulatory organ, and the onset of its function occurs relatively early in some teleost fishes. As such, any defects in kidney development and function are likely associated with a decreased ability to osmoregulate. Previous work has shown that early-life stage (ELS) zebrafish (*Danio rerio*) acutely exposed to *Deepwater Horizon* (DWH) crude oil exhibit transcriptional changes in key genes involved in pronephros development and function, as well as pronephric morphological defects and whole-animal osmoregulatory impairment. The objective of this study was to examine the acute effects of crude oil exposure during zebrafish ELS on pronephros function by assessing its fluid clearance capacity and glomerular filtration integrity. Following a 72-h exposure to control conditions, 20% or 40% dilutions of high-energy water-accommodated fractions (HEWAF) of DWH crude oil, zebrafish were injected into the common cardinal vein either with fluorescein-labeled (FITC) 70-kDa dextran to assess glomerular filtration integrity or with FITC-inulin to assess pronephric clearance capacity. Fluorescence was quantified after the injections at predetermined time intervals by fluorescence microscopy. The results demonstrated a diminished pronephric fluid clearance capacity and failed glomerular perfusion when larvae were exposed to 40% HEWAF dilutions, whereas only a reduced glomerular filtration selectivity was observed in zebrafish previously exposed to the 20% HEWAF dilution.

Keywords Crude oil · Pronephros · Glomerular filtration · Renal clearance · *Deepwater Horizon* · Zebrafish · Osmoregulation

Introduction

Large environmental disasters, such as the *Deepwater Horizon* (DWH) oil spill in 2010, have instigated the need to better understand the ecological consequences of these events (Joye et al. 2016). Numerous studies have investigated the lethal and sub-lethal effects of DWH crude oil exposures to aquatic organisms, especially teleost fishes (see Price and Mager 2020; Grosell and Pasparakis 2021; Murawski et al. 2021 and Takeshita et al. 2021 for recent reviews). Of the many constituents of crude oil, polycyclic aromatic hydrocarbons (PAHs) are believed to be the main drivers of toxicity (Forth et al. 2017). The most recognized sub-lethal effect associated with fish early-life stage (ELS) crude oil exposure is a cardiotoxic syndrome characterized by a suite of functional and morphological defects, the most common and prominent of which is the occurrence of pericardial and yolk sac edema (Incardona et al. 2014; Incardona and Scholz 2016). This edema is thought to arise in large part as a sequela to impaired cardiac output, likely due to reductions in heart rate or stroke volume and/or alterations to the heart's normal contractile rhythm (i.e., arrhythmias; Incardona et al. 2014). Furthermore, the reduced cardiac function during ELSs can have implications for the development of other organs, particularly those that rely on circulatory blood pressure and shear stress for proper vessel formation, such as the developing pronephros (Serluca et al. 2002; Drummond 2003; Incardona et al. 2004). Indeed, accumulating evidence

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at the transcript, morphological, and functional levels indicate likely alterations to normal pronephric development following teleost ELS crude oil exposure with implications for proper maintenance of osmotic homeostasis (Incardona et al. 2004; Xu et al. 2016; Bonatesta et al. 2022).

During larval stages, teleost fishes, including zebrafish (*Danio rerio*), use the pronephros as a functional kidney (Drummond, 2003). Once fully formed, the pronephros has a basic structure (Gerlach and Wingert 2013), comprised of a single glomerulus, two neck segments, two pronephric tubules, and two pronephric ducts that drain urine through a shared cloaca. The glomerulus is fully formed and functional at 48-h post-fertilization (hpf) in zebrafish (Gerlach and Wingert 2013). Its primary function is to filter the blood by preventing cells and large molecules from entering the subsequent pronephric segments (Kramer-Zucker et al. 2005b). Adjacent to the glomerulus are two neck segments, each of which are ciliated epithelial tubules that connect the filtration apparatus with the pronephric tubules and transport the filtrate from the glomerulus to the tubules (Wingert et al. 2007). The pronephric tubules are involved in osmoregulation via ion-transport proteins on the basolateral membrane (Drummond 2003) and, like the neck segments, are characterized by the presence of ciliated cells that are important for inducing fluid flow within the tubule lumen (Kramer-Zucker et al. 2005a). The last segment of the tubules meets the pronephric ducts, to which the filtrate is passed, and the last solutes are recovered. The two parallel ducts meet and fuse caudally before the cloaca (Gerlach and Wingert 2013), where excretion to the external environment occurs.

With respect to known crude oil effects on pronephros development, Bonatesta et al. (2022) recently demonstrated transcriptional and morphological defects associated with the pronephric glomerulus, neck segments, and tubules in DWH crude oil-exposed zebrafish larvae. Specifically, in acute (72 h) oil-exposed larvae, the neck segments were shortened or absent, and the tubules presented a straight shape instead of a typical convoluted shape (Bonatesta et al. 2022). These findings were consistent with previous work investigating the effects of zebrafish larvae acutely exposed to phenanthrene, a 3-ring PAH highly enriched among PAHs found within weathered crude oil (Incardona et al. 2004). Additionally, glomerulogenesis appeared to be altered by the modified mRNA expression of transcription factors involved in the formation of the filtration apparatus and the capillary tuft (Bonatesta et al. 2022). Notably, the pronephros requires a proper cardiovascular system to develop a functional glomerulus (Serluca et al. 2002); thus, the reduced cardiac function induced by crude oil exposure could also influence glomerulogenesis in exposed fish. In support of this, mutant zebrafish lacking a heartbeat failed to recruit podocytes to form a proper capillary tuft and, therefore, failed to form a proper filtration apparatus (Serluca et al. 2002).

As such, altered glomerulogenesis in response to crude oil exposure could conceivably result in reduced filtration properties of the pronephros, as evident by the leakage of larger molecules during glomerular perfusion (Hentschel et al. 2007). In more severe cases, failed glomerulogenesis might also result in reduced or inhibited glomerular perfusion and therefore reduced/inhibited renal clearance capacity in fish larvae with a subsequent induction of edema formation (Kramer-Zucker et al. 2005b; Perner et al. 2007). Furthermore, defects associated with the development of the pronephric tubules and neck segments could represent additional factors for a potential reduced renal clearance capacity (Kramer-Zucker et al. 2005a). Shortening of the cilia within these pronephric segments and reduced cilia beating frequency are known to induce pronephric morphological defects and inhibit the fluid clearance capacity of the pronephros (Kramer-Zucker et al. 2005a). The reduction of cilia structure and function induces pericardial and yolk sac edema in zebrafish larvae (Kramer-Zucker et al. 2005a), which is also a phenotypical defect highly observed in crude oil-exposed ELS fishes (Incardona and Scholz 2016). Hence, edema in crude oil-exposed larval fishes could indicate the presence of reduced or inhibited renal fluid clearance, as previously speculated (Incardona et al. 2004; Bonatesta et al. 2022). Interestingly, a recent study showed that DWH crude oil exposure induced pericardial and yolk sac edema in zebrafish larvae in a concentration-dependent fashion (Bonatesta et al. 2022). When these larvae were further osmotically challenged in hypoosmotic waters, the edema insult was exacerbated; contrarily, when osmotically challenged in waters near to isosmotic conditions, the differences in edema size were negated. These observations, coupled with the pronephros morphological defects (Bonatesta et al. 2022), suggest the need to better understand potential crude oil-induced functional defects in the developing pronephros.

Given that DWH crude oil exposures to ELS zebrafish induce clear pronephric morphological defects, we hypothesized that such defects would manifest in impaired pronephric function, namely glomerular filtration and renal clearance capacity, that likely contribute to the edema commonly observed in crude oil exposed ELS fish. Thus, the objective of this study was to examine the acute effects of 72-h crude oil exposure on glomerular filtration and renal clearance capacity of the pronephros in zebrafish larvae. We assessed these functional endpoints by quantifying a time course of renal clearance of fluorescein isothiocyanate-conjugated (FITC-) 70-kDa dextran and FITC-inulin (3–5 kDa), respectively. Dextran (70 kDa) was selected because of its inability to pass through the properly formed filtration apparatus of the glomerulus due to its large molecular size (Hentschel et al. 2007), while inulin was selected as it is easily filtered through the glomerulus due to a smaller size, and it is not reabsorbed by the pronephric tubules (Hentschel

et al. 2005; Rider et al. 2012). Based on these molecular properties, impaired integrity of the filtration apparatus of the glomerulus should result in clearance of 70-kDa dextran, while impaired glomerular perfusion and/or internal pronephric fluid flow should result in inulin persistence within the larvae.

Methods

Experimental design

Two separate tests were performed. The first test was performed to assess pronephric glomerular integrity using FITC-labeled 70-kDa dextran (more detail in the "Dextran injection" section). The second test was performed to assess pronephric clearance capacity using FITC-labeled inulin (3–5 kDa; more details in the "Inulin injection" section). Exposure and injection procedures were similar among tests unless indicated differently.

HEWAF preparation and analysis

Stock solutions of high-energy water-accommodated fractions (HEWAFs) of DWH slick oil from surface waters (OFS; 20,100,719-JUNIPER-001) were prepared daily with Embryo (E3) medium ($CaCl_2 \cdot 2H_2O$ 0.33 mM, $MgSO_4$ 0.33 mM, NaCl 5 mM, KCl 0.17 mM, in 1 L of Milli-Q water). HEWAFs were prepared daily at an oil loading rate of 1 g/L as previously described (Mager et al. 2014). Briefly, after mixing for 30 s on low in a blender, the HEWAF was transferred to a separatory funnel, capped, and covered from direct light for 1 h. Subsequently, the lower ~ 90% of the HEWAF stock solution was collected and used for the preparation of experimental solutions. With each HEWAF preparation, an initial sample of the 40% HEWAF dilution was collected and held at 4 °C until analysis. Extraction and GC/MS-SIM analysis of PAHs were performed by ALS Environmental (Kelso, WA) according to USEPA methods 3510C and 8270D, respectively. Reported ∑PAH concentrations represent the sum of 50 select PAH analytes (Table S1). Sum PAH concentrations for the 20% HEWAF were estimated by simply dividing the measured concentrations for the corresponding 40% HEWAF preparations by two. Previous studies have demonstrated that this approach provides a reasonable estimate of ∑PAH concentrations in diluted HEWAFs, particularly in the high range of HEWAF dilutions used in this study (Forth et al. 2017; Mager et al. 2017).

HEWAF exposure

Zebrafish (*Danio rerio*) embryos were obtained from the wild-type AB zebrafish brood stock at the University of North Texas (IACUC protocol #19–016) which was purchased from a local pet store (Fish n' Chirps Pet Center, Denton, TX). The brood stock was fed twice daily with TetraMin Tropical Flakes (Tetra, Blacksburg, VA) and maintained following standard husbandry conditions for zebrafish (14:10-h light/dark cycle, pH ~ 7.5, ~ 7.8 mg/L of dissolved oxygen, temperature ~ 28 °C; Lawrence 2007; Westerfield 2007). To obtain embryos, 6 breeding tanks (3 L) were set by placing 1 male and 1 female per tank. The two sexes were kept separated by a divider within the breeding tank overnight. At the start of the light cycle, the divider was removed, and the two sexes were able to breed for 15–20 min. Embryos were pooled and collected from multiple spawns (3–6) and assessed for quality and fertilization rate under a dissecting microscope. Spawns with low fertilization rate (< 85%) or frequent abnormalities (> 5%) were not used. HEWAF dilutions were prepared using E3 medium for nominal test solutions of 0%, 20%, and 40%. Nominal HEWAF dilutions were selected based on our previous study revealing morphological defects in the zebrafish pronephros (Bonatesta et al. 2022). Seventy-two-hour exposures were performed using 200 mL of test solution in 250-mL glass crystalizing dishes and initiated with the addition of zebrafish embryos at approximately 1 hpf. The duration of the HEWAF exposure was selected to ensure sufficient time for the onset of glomerular filtration, which in zebrafish is initiated at 48 hpf (Drummond 2003), and to facilitate direct comparison to the previous study using isolated PAHs that assessed morphology at 72 h of exposure (Incardona et al. 2004). Water changes (> 80%) were performed daily using dilutions of a freshly prepared HEWAF each day for the full experiment duration. Both tests had 3 replicates per treatment with 25 embryos/larvae per replicate. Exposures were performed in a Precision Scientific 818 Low Temperature Illuminated Incubator (SpectraLab Scientific Inc., Markham, Canada) set at 28 °C. Temperature, dissolved oxygen (DO), and pH were recorded daily using a YSI ProODO oxygen meter (YSI Incorporated, Yellow Springs, OH) for temperature and DO and an Orion Star A121 portable pH meter (ThermoFisher Scientific, Waltham, MA) for pH, each calibrated daily.

Injection solution preparation

For the first test, 0.5% (5 mg/ml) FITC-labeled 70-kDa dextran (ThermoFisher Scientific, Waltham, MA) was prepared in saline (0.9% NaCl), referred to henceforth as the "dextran solution." For the second test, 0.5% (5 mg/ml) FITC-inulin (Sigma-Aldrich, St. Louis, MO) was also prepared in saline, referred to henceforth as the "inulin solution." Subsequently, the inulin solution was dialyzed using a TUBE-O-DIALYZER (G-BIOSCIENCES, St. Louis, MO) dialysis tube with a membrane with a molecular weight cutoff of 1 kDa.

Injection preparation

The injection protocol was adopted by Hentschel et al. (2005, 2007) and Rider et al. (2012) with some minor modifications. Following the 72-h HEWAF exposure, zebrafish larvae (72 hpf) were briefly rinsed in E3 medium and then transferred into clean E3 medium prior to injection. Fifteen larvae were randomly selected per treatment (control 20% and 40% HEWAF) for the injection procedure. Larvae were anesthetized by MS-222 (100 mg/L buffered with $NaHCO_3$) prepared in E3 medium (henceforth referred to as the "anesthetic solution") approximately 15 min prior to the injections. Anesthetized larvae were placed laterally on a 2% agarose-E3 medium mold in anesthetic solution in preparation for the injection.

Dextran injection

Injections were performed at room temperature (24.4 °C) using a microinjector system (FemtoJet 4i and InjectMan 4; Eppendorf AG, Hamburg, Germany) under a Leica S9D stereo microscope (Leica, Wetzlar, Germany). The treatment groups were injected in the following order: 20% HEWAF larvae, control larvae, and 40% HEWAF larvae. To assess glomerular filtration integrity, larvae were injected into the common cardinal vein (CCV) with 4.2 nL of the dextran solution. Injected larvae were then transferred into clean E3 media and placed in the incubator, while the injections for the other treatments were performed. Subsequently, larvae were re-anesthetized in anesthetic solution and mounted laterally in a 24-well culture plate (one larva per well) in 3% methylcellulose prepared with E3 medium. Proper injection (Fig. 1A) was assessed by observing perfusion of the dextran solution throughout the vasculature via fluorescent microscopy on an EVOS FL-inverted microscope (ThermoFisher Scientific, Waltham, MA) using a GFP channel. Larvae that expressed fluorescence singularly in the pericardial area or yolk sac were considered miss-injected and not imaged. The control, 20% HEWAF, and 40% HEWAF treatments were comprised of 11, 10, and 9 larvae, respectively. Pictures were taken using the EVOS FL-inverted microscope with the same microscope settings. After the pictures were taken, larvae were rinsed in E3 medium and then placed in clean E3 medium in the incubator. Following the same steps, pictures were captured at 3, 6, 12, 24, 48, and 72 h post-injection (hpi). Mortality (if any) was assessed throughout the testing period. At the end of the test, larvae were euthanized with an overdose of MS-222.

Inulin injection

Injection procedures were similar to the dextran solution injection protocol, with a few modifications. To assess renal clearance capacity, larvae were injected into the CCV with 4.2 nL of dialyzed inulin solution. During this test, larvae were mounted laterally in a 24-well culture plate (one larva per well) in 3% methylcellulose prepared with E3 medium right after the injection. Correct injection was assessed as previously described. The control, 20% HEWAF, and 40% HEWAF treatments were comprised of 13, 9, and 10 larvae, respectively. Pictures were taken using the same microscope within the first 15–20-min post-injection. After the pictures were taken, larvae were rinsed in E3 medium and placed in clean E3 medium in the incubator. Following the same steps, pictures were captured at 0.2, 1, 3, 6, and 24 hpi.

Image analysis

Pictures were converted into grayscale using ImageJ NIH software. Mean grayscale fluorescence intensity was quantified around the head and pericardial area per each larva (see Fig. 1B as an example of area selected) at each time point using ImageJ. This specific area was selected for analysis to exclude potential unreliable intensity measurements

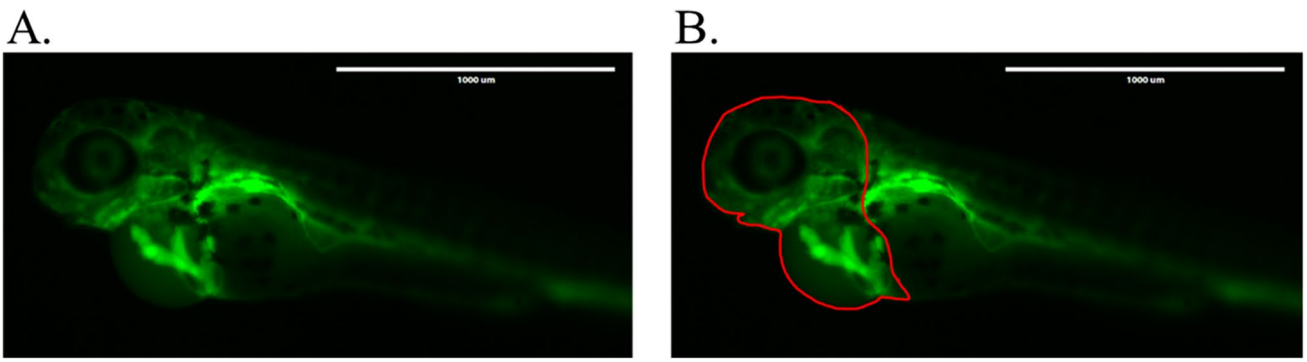

Fig. 1 Example of proper injection by observing the FITC-dextran solution moving through the circulatory system in the zebrafish larva. **A** 75-hpf zebrafish larva (3 hpi) that was previously exposed to 20% HEWAF (162 µg/L ΣPAH) for 72 h and was injected with 4.2 nL of 5% FITC-dextran solution. **B** The area selected for mean fluorescence quantification

associated with fluorescent compounds trapped in the yolk sac and/or site of injection. Similarly, background mean grayscale fluorescence intensity was quantified per image selecting a random area outside of the fish larva. Background mean fluorescence intensity of each specific image was subtracted from the mean fluorescence intensity quantified for the respective fish larva, and this value was used for further analysis (henceforth referred to as "mean grayscale intensity"). In the few instances where the mean fluorescence intensity resulted in a slightly negative value (e.g., when full clearance of the fluorescent molecule occurred), the mean fluorescence intensity was assumed to be zero. Mean fluorescence intensity percentage was calculated for each larva at each time point relative to the mean fluorescence intensity quantified during the first time point imaged (3 hpi for the dextran test and 0.2 hpi for the inulin test), with the first time point analyzed considered 100%. Pericardial and yolk sac edema area was measured using the same images according to the protocol provided by Edmunds et al. (2015) using ImageJ NIH software. Measurements were taken for 72-hpi zebrafish larvae (144 hpf) at the end of the first test (dextran injection) and for 24-hpi zebrafish larvae (96 hpf) at the end of the second test (inulin injection).

Statistical analysis

Statistical analyses were performed using SigmaPlot 12.3 (Systat Software, Inc., San Jose, CA). For both tests, ANOVA on Ranks was performed to analyze differences in mean fluorescence intensity and mean fluorescence intensity percentage at each time point per treatment, as well as to analyze total edema area. Differences were considered significant at $P < 0.05$.

Results

Water parameters, PAHs analysis, and survival rate

Initial and final water parameters (temperature, pH, and DO) measured daily from each test chamber during the HEWAF exposure and the initial water parameter of the post-injection control E3 medium are presented as means ± SEM per treatment (Table S2). HEWAF concentrations are presented as the means ± SEM of initial values of Σ50 PAH concentrations (Table S2). The 40% HEWAF PAH profiles are presented in Table S1. For the first test, the measured ΣPAH concentration for the 40% HEWAF was 324.8 ± 27.0 µg/L, and therefore the estimated ΣPAH for the 20% HEWAF was 162.4 ± 13.5 µg/L. For the second test, the measured and estimated concentrations were 323.4 ± 24.2 µg/L and 161.7 ± 12.1 µg/L, respectively. Survival during the HEWAF exposures is presented in Table S3. No mortality was observed in injected larvae during the dextran injection test. One larva previously exposed to 40% HEWAF died at 24 hpi during the inulin injection test and, consequently, was excluded for fluorescence intensity analysis during that time point.

Dextran injection

The injecting needle was calibrated and used for all injections performed for the three treatments without the need for changing or recalibration. FITC-dextran mean grayscale intensity was significantly different in zebrafish larvae previously exposed to 20% HEWAF (162.4 ± 13.5 µg/L ΣPAH) compared to larvae previously exposed to 40% HEWAF (324.8 ± 27.0 µg/L ΣPAH) and to control larvae at all time points (Fig. 2). No difference in mean fluorescence intensity percentage was observed across treatments at any time point analyzed (Fig. 3).

Inulin injection

The same needle was used for the control and the 40% HEWAF (323.4 ± 24.2 µg/L ΣPAH) pre-exposed group. During the injection of the 20% HEWAF (161.7 ± 12.1 µg/L ΣPAH) pre-exposed group, the needle was partially clogged (not noticed during the injection), resulting in a decreased injected volume of the inulin solution. Consequently, the mean fluorescence intensity for the 20% HEWAF pre-exposed group was not used for the analysis, as no direct comparison with the other groups could be made. However, the mean fluorescence intensity percentage was used as it represents the percentage change over time relative to the first time point imaged. FITC-inulin mean fluorescence

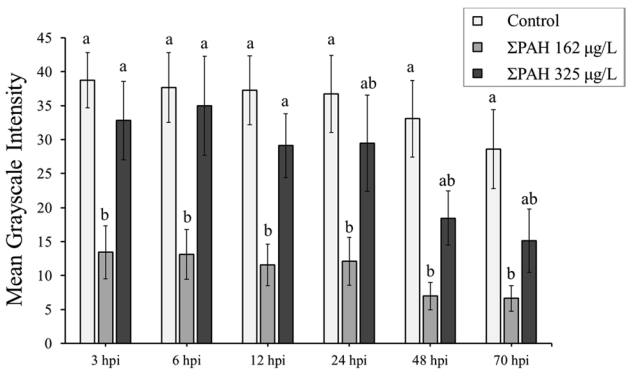

Fig. 2 Time course of mean grayscale intensity measured for FITC-dextran. Prior to injection, zebrafish larvae were exposed to either control medium, 20% HEWAF (162 µg/L), or 40% HEWAF (325 µg/L ΣPAH). Injections were performed at 72 hpf, and larvae were transferred into control medium for the rest of the test. Error bars represent ± SEM ($n = 9$–11). Differences were considered statistically significant within a time point at $P < 0.05$ (bars sharing letters do not significantly differ)

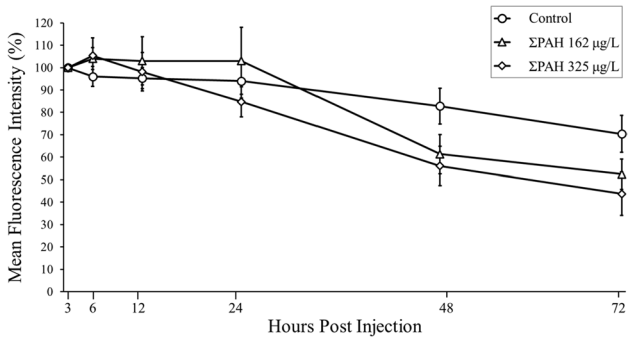

Fig. 3 Time course of mean fluorescence intensity measured for FITC-dextran as the percentage relative to the first time point analyzed (3 hpi). Prior to injection, zebrafish larvae were exposed to either control medium, 20% HEWAF (162 µg/L), or 40% HEWAF (325 µg/L ΣPAH). Injections were performed at 72 hpf, and larvae were transferred into control medium for the rest of the test. Error bars represent ± SEM ($n = 9–11$)

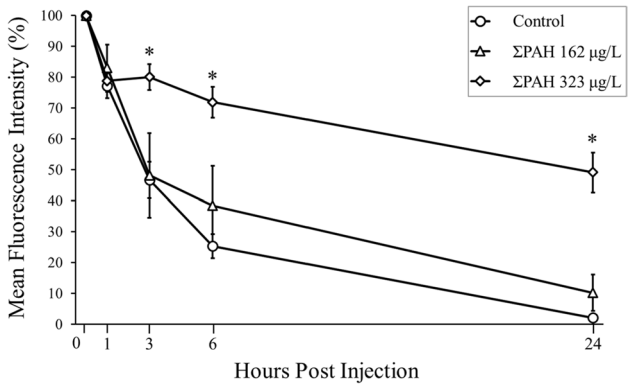

Fig. 4 Time course of mean fluorescence intensity measured for FITC-inulin as the percentage relative to the first time point analyzed (0.2 hpi). Prior to injection, zebrafish larvae were exposed to either control medium, 20% HEWAF (162 µg/L), or 40% HEWAF (325 µg/L ΣPAH). Injections were performed at 72 hpf, and larvae were transferred into control medium for the rest of the test. Error bars represent ± SEM ($n = 9–14$). $P < 0.05$ (* = statistically different than control and other treatment)

intensity for the zebrafish larvae previously exposed to 40% HEWAF was significantly different than control larvae at 3, 6, and 24 hpi (Figure S1). Mean fluorescence intensity percentage for the zebrafish larvae previously exposed to 40% HEWAF was significantly higher from the other treatments at 3, 6, and 24 hpi indicating reduced inulin solution clearance (Fig. 4).

Pericardial and yolk-sac edema

In both tests, following the 72-h HEWAF exposure and several hours post-injections (72 h for the dextran injection and 24 h for the inulin injection), zebrafish larvae that were previously exposed to the 40% HEWAF (365 and 362 µg/L ΣPAH) presented a significantly enlarged edema area compared to control and the other treatment (Fig. 5). Morphologically, the edema was characterized as severe whole-body edema in both instances.

Discussion

Previous studies have demonstrated the adverse effects of acute early-stage DWH crude oil or individual PAH exposure on the development of the teleost kidney (Incardona et al. 2004; Xu et al. 2016, 2017; Bonatesta et al. 2022). These findings were observed primarily through analyzing molecular and morphological endpoints. The current study is the first to have assessed functional defects of the pronephros in larval fish induced by ELS crude oil exposure. Specifically, we have demonstrated that pronephric function of zebrafish larvae is impaired by DWH crude oil exposure in a concentration-dependent manner, with failed glomerular perfusion and renal clearance at higher concentrations and altered glomerular filtration properties at lower concentrations. Coupled with the molecular and morphological defects previously observed, these results aid in providing a more complete picture of the detrimental effects of ELS crude oil exposure on teleost kidney development and function.

The onset of glomerular filtration, and therefore the presence of a functional glomerulus, occurs soon after 48 hpf, when podocytes fully surround the capillary tuft, which is made of fenestrated endothelial cells (Gerlach and Wingert 2013). In between these two cell layers, there is a glomerular basement membrane (GBM) which functions as a filter for the incoming blood (Gerlach and Wingert 2013). In addition to the GBM, the slit diaphragm, which is a junctional complex of podocyte foot processes, functions as an additional filtration barrier by preventing cells and larger molecules from moving into the subsequent pronephric segments (Kramer-Zucker et al. 2005b). Following the onset of glomerular filtration, the filtrate is formed, and it is moved through the two neck, tubule, and duct regions by motile cilia to get excreted (Kramer-Zucker et al. 2005a). Morphologically, for the next several days of development, there is an expansion of each pronephric tubule domain with a rostral collective cell migration driven by the fluid flow within the lumen (Kramer-Zucker et al. 2005a; Freund et al. 2012), emphasizing the need for a proper internal fluid flow. Consequently, the proximal segment of the tubules becomes more convoluted over time (Gerlach and Wingert 2013). During these life stages, excretion, especially of fluid, occurs through the functional pronephros and, in part, by the epidermis, especially since the gills do not become functional until 7 dpf in zebrafish larvae (Rombough 2002; Drummond 2003). Any implication to these tightly regulated

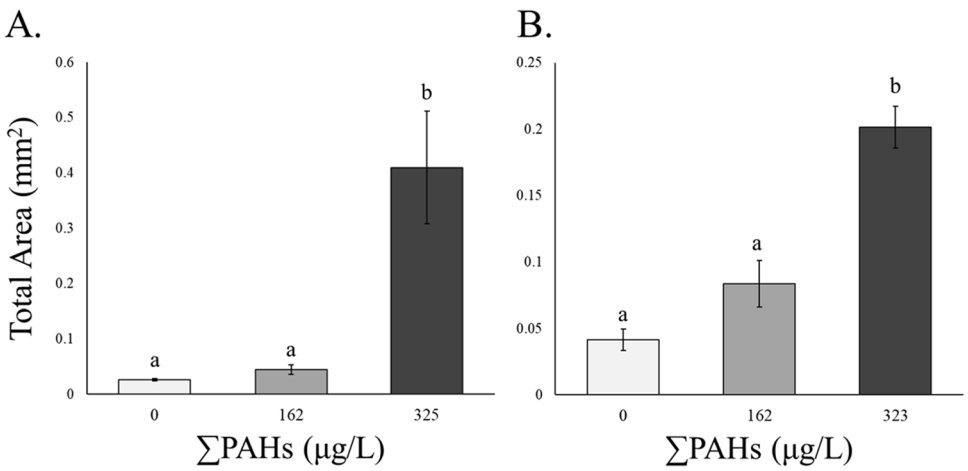

Fig. 5 Total area of pericardial and yolk sac edema size in zebrafish larvae represented as means ± SEM per treatment. **A** 72-hpi larvae (144 hpf) previously exposed to crude oil from ~ 1 to 72 hpf and injected with FITC-labeled 70-kDa dextran; **B** 24-hpi larvae (96 hpf) previously exposed to crude oil from ~ 1 to 72 hpf and injected with FITC-labeled inulin. Differences were considered significant at $P < 0.05$ (bars sharing letters do not significantly differ from each other)

developmental processes could result in both morphological and functional defects associated with the pronephros (Bonatesta et al. 2022).

Developmental defects associated with the glomerulus can result in the altered structure or absence of the filtration system (i.e., GBM and slit diaphragm; Kramer-Zucker et al. 2005b; Hentschel et al. 2007). In more extreme cases, it can result in the failed formation of the capillary tuft and, therefore, lack of glomerular perfusion (Kramer-Zucker et al. 2005b; Perner et al. 2007). As previously mentioned, 70-kDa FITC-dextran was selected to assess the filtration integrity of the glomerulus due to its large molecular size and inability to be properly filtered by the glomerulus (Hentschel et al. 2007), as was confirmed by the control group of the current study (Figs. 2 and 3). Bonatesta et al. (2022) previously showed that glomerulogenesis of the zebrafish is affected by ELS crude oil exposure. Therefore, we speculated that the perfusion of the glomerulus and its filtration capacity were altered by DWH crude oil exposure. Our results revealed no statistical differences in the mean fluorescence intensity percentages among the treatments tested over the 72 hpi (Fig. 3). However, the mean fluorescence intensity calculated for zebrafish larvae that were previously exposed to 20% HEWAF was significantly lower at each time point throughout the test, starting at 3 hpi (Fig. 2). Interestingly, we did not see the same response in larvae that were previously exposed to 40% HEWAF (Fig. 2). This likely indicates that the larvae exposed to 20% HEWAF excreted the majority of the injected FITC-dextran within the first 3 h of the injection and, therefore, that the filtration selectivity of the glomerulus was negatively affected by crude oil exposure (supported later by the inulin test). The results differed for the zebrafish larvae previously exposed to 40% HEWAF, as no difference of mean fluorescence intensity was observed compared to the control group (Fig. 2). This outcome is most likely due to a more severe concentration-dependent crude oil-induced effect on the development of the pronephros, which can be characterized by the lack of glomerular perfusion and, therefore, the inhibition of renal clearance capacity. This premise can be confirmed by analyzing the morphological defects in zebrafish larvae following crude oil exposure, as described in our previous study (Bonatesta et al. 2022). Specifically, zebrafish larvae exposed to 467 µg/L ΣPAH for 72 hpf (the same time point at which the injection was performed during the current study) presented an apparent reduction in *vegfr2* pronephric expression by whole-mount in situ hybridization, which is the vascular endothelial growth factor receptor expressed by endothelial cells in the glomerulus and is involved in the formation of the capillary tuft. Furthermore, the same larvae presented failed convolution of the proximal tubules and a shortening/lack of the neck segments, which are signs of reduced or absent fluid flow within the pronephros (Kramer-Zucker et al. 2005a; Vasilyev et al. 2009), emphasizing again a likely reduction or lack in glomerular filtration. Importantly, these morphological defects were not present in 72 hpf zebrafish larvae exposed to a lower ΣPAH concentration (234 µg/L), which presented the typical convoluted shape of the tubules, an indicator of proper internal fluid flow (Kramer-Zucker et al. 2005a; Vasilyev et al. 2009).

Based on these observations, further confirmation of proper glomerular filtration was warranted. Therefore, we examined the pronephric clearance capacity of DWH crude oil-exposed zebrafish larvae using shorter time intervals. Injecting FITC-inulin resulted in a significantly higher mean fluorescence intensity percentage starting at 3 hpi in larvae previously exposed to 40% HEWAF compared to the other treatments indicating significantly reduced excretion (Fig. 4). Conversely, larvae that were previously exposed to 20% HEWAF presented similar excretion capacity as in control fish, characterized by ~ 50% FITC-inulin excretion at around 3 hpi and almost complete excretion at 24 hpi (Fig. 4). This result likely confirms our suspicion that the FITC-dextran was excreted within the first 3 hpi in zebrafish

larvae previously exposed to 20% HEWAF, likely due to a defective glomerular filtration apparatus. However, the reduced ability to excrete FITC-inulin by larvae previously exposed to 40% HEWAF provides evidence of likely reduced glomerular perfusion and inhibited pronephric fluid flow. Furthermore, by increasing the fluorescence light intensity of the microscope, it was possible to observe that some FITC-inulin was adsorbed/absorbed by the tubules in control larvae (Figure S2), confirming the excretion route of this compound through the pronephros (Hentschel et al. 2005; Rider et al. 2012).

Notably, the pronephros requires a proper cardiovascular system to develop a functional glomerulus (Serluca et al. 2002). Within the first week of development, zebrafish larvae can survive without a functional heart (Serluca et al. 2002; Burggren et al. 2017), implying that the function of the heart during ELSs is not the same as that in adult fish (Burggren et al. 2017). Instead, it has been proposed that the importance of vascular blood pressure and shear stress are integral to organ development during ELSs (Serluca et al. 2002; Freund et al. 2012), as is the case for the glomerulus. Serluca et al. (2002) has highlighted the importance of hemodynamic forces to glomerulogenesis in zebrafish. As previously reported, a fully functional glomerulus occurs ~ 48 hpf when podocytes merge in the middle to recruit endothelial cells from the dorsal aorta and form a capillary tuft (Drummond 2003). The obstruction (genetic, pharmacological, or physical) of blood flow through the dorsal aorta results in failed glomerular formation as the podocytes fail to merge medially, and there is no formation of a capillary loop (Serluca et al. 2002). Crude oil-induced cardiotoxicity is believed to induce secondary effects during fish development (Incardona et al. 2004); thus, the reduced cardiac function induced by crude oil exposure needs to be considered not only as a potential factor for failed glomerulogenesis but also as a reducing factor of glomerular perfusion in crude oil exposed fish (Hentschel et al. 2007; Rider et al. 2012). Effects on heart function (i.e., heart rate and stroke volume) were not assessed during the current study. However, previous studies have revealed reductions in heart rate in zebrafish larvae exposed to crude oil. Specifically, 96-h exposure to a concentration as low as 75 µg/L ΣPAH elicited reduced heart rate even after 24 h in clean water (Bonatesta et al. 2022). Additionally, a similar reduction in heart rate was observed in zebrafish larvae exposed for 62 h to 64 µg/L ΣPAH, but with no effect on stroke volume or cardiac output (Magnuson et al. 2020). Even though these exposures are not directly comparable to the current study due to the length and the concentrations used, it is likely that the crude oil-exposed zebrafish larvae described herein presented more severe cardiac defects due to the higher ΣPAH concentrations used (162 and 325 µg/L). Therefore, reduced perfusion of the glomerulus due to reduced cardiac function likely explains in large part the reduced clearance observed during the current study, especially in larvae exposed to 40% HEWAF.

However, it is important to mention that proper nephric cilia beat frequency and structure are also necessary for renal clearance, which is achieved even with the pharmacological stoppage of cardiac function (Kramer-Zucker et al. 2005a). Significantly for the pronephric function, cilia modification induces adverse effects on renal clearance capacity in 72–84 hpf zebrafish, as delayed or no clearance has been observed under this scenario (Kramer-Zucker et al. 2005a). Modification (shortening or reduced beating) and disruption of the cilia (not only nephric cilia) in zebrafish embryos/larvae have also been associated with similar phenotypical outcomes as in PAH-exposed ELS fish (Incardona et al. 2004; Kramer-Zucker et al. 2005a), namely kidney cyst formation, hydrocephalus and pericardial edema highly evident at 72 hpf (which becomes a more generalized edema by 120 hpf), and spinal curvature. Although cilia defects were not assessed in the current study, when coupled with the known cardiotoxic effects, they could conceivably contribute to the reduced/inhibited renal clearance capacity in crude oil-exposed fish.

Finally, another phenotypical defect likely attributed at least in part to crude oil toxicity to the pronephros is pericardial and yolk-sac edema, which evolves into a more general edema in later stages (Marty et al. 1997; Jung et al. 2013; Incardona and Scholz 2016; Bonatesta et al. 2022). Given the importance of pronephros clearance capacity during fish ELSs, any factors affecting the excretory ability of the pronephros could result in fluid accumulation in fish larvae (Kramer-Zucker et al. 2005b; Perner et al. 2007; Bonatesta et al. 2022). One of these factors could be related to pronephros morphological defects during development, as was previously explored (Bonatesta et al. 2022). The results of our study have indeed shown that renal clearance is inhibited in DWH crude oil-exposed zebrafish larvae, especially when exposed to a higher ΣPAH concentration during both tests. Phenotypically, zebrafish larvae that were previously exposed to the lower ΣPAH concentrations did not present an edematous pericardial and yolk-sac area following the HEWAF exposure and injections, in direct contrast to the larvae that were previously exposed to the highest ΣPAH concentrations (Fig. 5), which were characterized by whole-body edema at the end of the fluorescence analysis in both tests. These phenotypical differences further confirm the importance of renal clearance in edema formation due to the inability to excrete water. It is important to remember that even though renal clearance was observed in zebrafish larvae exposed to lower ΣPAH concentration, glomerular filtration capacity was negatively affected by the exposure and thus has the potential to induce latent osmoregulatory effects.

Conclusion

Pronephros function is impaired by acute DWH crude oil exposure in ELS zebrafish. Specifically, larvae exposed to high ΣPAH concentrations have a reduced/inhibited renal clearance capacity, which can be driven by failed glomerular perfusion or lack of internal tubule fluid flow. On the other hand, larvae that were exposed to lower ΣPAH concentrations exhibited proper renal clearance but defective glomerular filtration integrity. Coupled with the molecular and morphological defects associated with DWH crude oil exposure, these results provide significant insight into the formation of edema in larval fish and functional defects associated with the pronephros. Assessing functions of specific osmoregulatory structures in small organisms, such as zebrafish embryos and larvae, can be somewhat challenging. However, the techniques used during the current study proved well-suited to assess renal clearance capacity and glomerular filtration selectivity in larval fish.

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Declarations

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