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Early life-stage *Deepwater Horizon* crude oil exposure induces latent osmoregulatory defects in larval red drum (*Sciaenops ocellatus*)

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ABSTRACT

Crude oil is known to induce developmental defects in teleost fish exposed during early-life stages (ELSs). A recent study has demonstrated that zebrafish (*Danio rerio*) larvae acutely exposed to *Deepwater Horizon* (DHW) crude oil showed transcriptional changes in key genes involved in early kidney (pronephros) development and function, which were coupled with pronephric morphological defects. Given the osmoregulatory importance of the kidney, it is unknown whether ELS effects arising from short-term crude exposures result in long-term osmoregulatory defects, particularly within estuarine fishes likely exposed to DWH oil following the spill. To address this knowledge gap, an acute 72 h exposure to red drum (*Sciaenops ocellatus*) larvae was performed using high-energy water-accommodated fractions (HEWAFs) of DWH weathered oil to analyze transcriptional changes in genes involved in pronephros development and function by quantitative PCR. To test the latent effects of oil exposure on osmoregulation ability, red drum larvae were first exposed to HEWAF for 24 h. Larvae were then reared in clean seawater for two weeks and a 96 h acute osmotic challenge test was performed by exposing the fish to waters with varying salinities. Latent effects of ELS crude oil exposure on osmoregulation were assessed by quantifying survival during the acute osmotic challenge test and analyzing transcriptional changes at 14 dpf. Results demonstrated that ELS crude oil exposure reduced survival of red drum larvae when challenged in hypoosmotic waters and that latent transcriptional changes in some target pronephric genes were evident, indicating that an affected kidney likely contributed to the increased mortality.

1. Introduction

In 2010, the *Deepwater Horizon* (DWH) oil spill occurred in the Gulf of Mexico (GoM) when the Macondo oil well blowout released around 5 million barrels of crude oil, establishing it as one of the largest oil spills in history (Eckle et al., 2012). While natural seepages of hydrocarbons occur within the GoM, it has never experienced a release of such grave magnitude (Eckle et al., 2012). The spill coincided with the spawning of many pelagic fishes of ecological and economic importance (Rooker et al., 2013), representing a high risk to the fish in the GoM (Grosell and Pasparakis, 2021), especially to sensitive early-life stages (ELSs) (Pasparakis et al., 2019). A suite of crude oil induced effects during ELSs have been well characterized, such as the effects on heart development and function (Edmunds et al., 2015; Khursigara et al., 2017), eye development (Magnuson et al., 2018; Magnuson et al., 2020), ammonia and urea excretion (Wang et al., 2019), and cholesterol biosynthesis (McGruer et al., 2019; Price et al., 2022). However, there are still many unknowns, as many of these studies have focused solely on the proximate acute effects, whereas far fewer studies have investigated latent or chronic effects following transient ELS exposures (e.g., Brown-Peterson et al., 2015; Bautista et al., 2020).

In ELSs, most crude oil-induced sub-lethal effects are associated with cardiac development and function, referred to as cardiotoxicity (Incardona et al., 2004). It is believed that the induced cardiotoxicity causes secondary effects in the exposed fish (e.g., pericardial and yolk sac edema) and has potential implications for proper development of other organs (Incardona et al., 2004). Notably, morphological defects are evident in teleost early-stage kidney (i.e., pronephros) following exposure to DWH crude oil (Bonatesta et al., 2022) and to individual polycyclic aromatic hydrocarbons (PAHs) (Incardona et al., 2004; Lo et al.,

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2014), which are considered the major drivers of crude oil toxicity (Forth et al., 2017). Moreover, multiple lines of transcriptional evidence from several fish species point to alterations in pronephros development and function (Xu et al., 2016; Xu et al., 2017; Bonatesta et al., 2022). Given the osmoregulatory role of the kidney (Drummond, 2003), any potential negative outcome in its development and function could represent an adverse condition to the organism, potentially leading to mortality.

Teleost fishes osmoregulate by the cooperation of multiple structures: gills, gastrointestinal tract, skin, urinary bladder, and kidney (Marshall and Grosell, 2005). The role that these organs have in osmoregulation is usually dictated by the salinity concentration in the water (Larsen et al., 2014; Marshall and Grosell, 2005). Thus, to maintain internal osmotic homeostasis, teleost fishes adopt various osmoregulatory processes to compensate against the osmotic water gain/diffusive ion loss (hyper-osmoregulation) or osmotic water loss/diffusive ion gain (hypo-osmoregulation) when inhabiting hypoosmotic or hyperosmotic waters, respectively (Marshall and Grosell, 2005). Specific to teleost fishes inhabiting hypoosmotic freshwater, the kidney is crucial for generating a constant flow of highly dilute, hypotonic urine to discard diffusively gained water, as well as to reabsorb ions from the filtrate to compensate for the diffusive ion loss (McDonald, 2007). By contrast, in hyperosmotic saltwater the kidney is necessary to conserve water to compensate against the diffusive water loss, and to excrete divalent ions (such as Mg^{2+} and SO_4^{2-}; McDonald, 2007). In areas where saltwater and freshwater are mixed, such as estuaries and other brackish waters, salinity can vary dramatically on a diel scale (Montague and Ley, 1993; Barletta et al., 2005), presenting significant osmotic stress to the organisms. If an organism is unable to cope with this shift, the salinity stress can interfere with various biological processes and can cause mortality (Iso et al., 1994; Kültz, 2015; Komoroske et al., 2016).

The frequency and the severity of salinity shifts are increasing in many locations due to anthropogenic activities (Komoroske et al., 2016; Baker et al., 2017). In the GoM during the DWH spill, large amounts of river water was intentionally released into Black Bay/Breton Sound and Barataria Bay, Louisiana (USA) as a response action to prevent crude oil from reaching the coast (Baker et al., 2017). As a result, estimated salinities fell below 3 ppt and 5 ppt for over 19 and 30 consecutive days, respectively, between May and September 2010 (Rose et al., 2014; McDonald et al., 2015). While the decrease in salinity alone could have been detrimental for several species (Rose et al., 2014), the additional presence of crude oil was fatal for some species that inhabit the bay, such as oysters (*Crassostrea virginica*) (Baker et al., 2017) and brown shrimp (*Farfantepenaeus aztecus*) (Powers and Scyphers, 2016).

Red drum (*Sciaenops ocellatus*) is an ecologically and economically important euryhaline fish species found along the coasts and estuaries of the GoM (Wilson and Nieland, 1994). Red drum spawn near inlets from August to October, producing transparent, positively buoyant embryos (Wilson and Nieland, 1994). Red drum spend a significant amount of their early life in bays and estuaries, and therefore possess well-equipped osmoregulatory mechanisms for tolerating significant salinity shifts (Watson et al., 2014; Ern and Esbaugh, 2018; Martin and Esbaugh, 2021). Given the location and timing of the DWH spill, red drum very likely experienced crude oil exposure. Crude oil toxicity to red drum has been previously examined (Khursigara et al., 2017; Xu et al., 2017; Magnuson et al., 2018), and the larval stage appears to be highly sensitive to crude oil exposure (Alloy et al., 2017; Pasparakis et al., 2019; Bonatesta et al., 2020). Importantly, red drum larvae exposed to DWH crude oil showed the characteristic symptoms of cardiovascular toxicity (Khursigara et al., 2017), including pericardial and yolk-sac edema (Xu et al., 2017). Such edema is likely due, at least in part, to impaired kidney formation and function, which may be a direct sequalae of cardiotoxicity (Bonatesta et al., 2022). In any event, the euryhaline nature of red drum make them an ideal model to study the potential effects of crude oil exposure on the ability of estuarine fishes to osmoregulate within fluctuating osmotic environments, especially during ELSs.

Recent evidence has revealed that exposure to relatively high concentrations of DWH crude oil alters the transcriptional responses and impairs morphological development of the pronephros in zebrafish (*Danio rerio*) larvae (Bonatesta et al., 2022); however, it is unknown if environmentally relevant concentrations would induce similar effects in an environmentally relevant species. Furthermore, it is unknown whether effects arising from short-term exposures result in long-term or latent effects on osmoregulatory function. Therefore, the objectives of this study were two-fold: (1) determine the latent effects of crude oil exposure on the ability of red drum larvae to survive a range of osmotic challenges following a two-week period of recovery from crude oil exposure in control water, and (2) to analyze the morphological and transcriptional responses in the pronephros of red drum larvae exposed to DWH crude oil, focusing on genes involved in pronephros development that were previously shown to be altered in crude oil exposed zebrafish.

2. Methods

2.1. Experimental design

Two separate tests were performed. During the first test, a 24 h high-energy water accommodated fractions (HEWAF) exposure was performed from ~12 to 36 h post-fertilization (hpf); subsequently, the fish were raised until 14 d post fertilization (dpf) in clean water in preparation for the osmotic challenge tests (see Osmotic challenge test section). During the second test, a 72 h HEWAF exposure was performed from ~12 to 84 hpf to collect red drum larvae for qPCR analysis at various time points (see Sample collection for cDNA synthesis section).

2.2. HEWAFs preparation and analyses

Naturally weathered oil was collected from a slick in the GoM on June 29th, 2010 from the hold of barge number CT02404 (referred to as OFS). Stock solutions of HEWAFs were prepared with sterilized seawater (35 ppt). HEWAFs were prepared at an oil loading rate of 1 g/L as previously described (Mager et al., 2014). Briefly, each WAF was mixed for 30 s on low in a blender, transferred into a separatory funnel and capped. After 1 h, the lower ~90 % of the WAF stock solution was collected and used for the preparation of experimental solutions. A sample of the initial diluted solution was collected and held at 4 °C until analysis. ALS Environmental (Kelso, WA) performed the extraction and GC/MS-SIM analysis of PAHs according to USEPA methods 3510C and 8270D, respectively. Reported ∑PAH concentrations represent the sum of 50 select PAH analytes (Table S1).

2.3. Osmotic challenge test

For the first exposure, red drum embryos were collected from a brood stock maintained at the Texas Parks and Wildlife – CCA Marine Development Center in Corpus Christi, Texas, USA and transported to the University of Texas Marine Science Institute (IACUC protocol # AUP-2018-00231). The embryos (~ 11 hpf) were treated with formalin (1 ppt) for 1 h and rinsed before being transferred either into control seawater (35 ppt) or 1 % OFS HEWAF for 24 h until 36 hpf in a 9 L glass pickle jar (<5000 embryos per jar). This procedure was adapted from Magnuson et al. (2018), which used a similar procedure to explore larval ocular function following embryonic oil exposure. The 1 % HEWAF dilution was also selected based on Magnuson et al. (2018). After the exposure, embryos were rinsed and transferred to 164 L conical mesocosms containing sterilized seawater (35 ppt) until 14 days post fertilization (dpf). The fish started feeding at 3 dpf (72 hpf) and were fed enriched rotifers (0.3 g of Algamac 3050 per 1×10^6 rotifers) once daily until 10 dpf. On day 10 they were fed enriched rotifers and unenriched artemia once. On day 11 they were fed enriched rotifers and enriched

artemia once (0.2 g of Algamac 3050 per 1000 artemia). Starting on day 12 and throughout the osmotic challenge test they were fed enriched artemia once daily. At 14 dpf, 10 red drum larvae from each treatment (previously exposed to control or 1 % HEWAF) were transferred into a 1 L polypropylene beaker containing 800 mL of water with a salinity of 30, 18, 4, or 2 ppt (4 replicates per salinity). The 30 and 18 ppt waters were considered hyperosmotic, while 4 and 2 ppt waters were considered hypoosmotic relative to the internal osmolarity of the fish, which is approximated to be 10 ppt (Watson et al., 2014). The duration of the osmotic challenge test was 96 h, spanning from 14 dpf to 18 dpf. Water parameters (pH, salinity, temperature, dissolved oxygen) were recorded daily in each test chamber, and water changes (>80 %) were performed daily. Larvae were fed 1 h prior to daily water changes. Percent survival in each test chamber was quantified at 4, 16, 24, 48, 72 and 96 h. All remaining fish were euthanized by overdose of MS-222 (250 mg/L buffered with 500 mg/L $NaHCO_3$). Water parameters (temperature, dissolved oxygen, pH) were measured daily.

2.4. Sample collection for cDNA synthesis

For the second experiment, a 72 h static HEWAF exposure was performed. Red drum embryos/larvae were exposed to either 1 % OFS HEWAF or control seawater. Exposures were performed in 1 L glass beakers filled with 1 L solution per treatment loaded with 40 embryos/larvae each, starting at 12 hpf until 84 hpf (12 replicates per treatment). Water parameters (temperature, dissolved oxygen, pH) were measured daily. Whole larvae were randomly collected for mRNA isolation at 36, 60 and 84 hpf for each treatment (4 replicates of 20–40 larvae). Additionally, 14 dpf red drum larvae were collected prior to the osmotic challenge test from the first 24 h HEWAF exposure for mRNA isolation. Specifically, 5 replicates of 10 larvae were sampled per concentration at 14 dpf. Larvae were euthanized by overdose of MS-222 (250 mg/L buffered with 500 mg/L $NaHCO_3$), stabilized and stored in RNAlater (Invitrogen, Waltham, MA) solution at 4 °C. Samples were later homogenized with a tissue disperser (IKA Works, Wilmington, NC) in TRIzol® (Life Technologies, Carlsbad, CA). Total RNA isolation was performed with the Maxwell® 16 Instrument and the corresponding Maxwell® 16 LEV simplyRNA Tissue Kit (Promega Corporation, Madison, WI) following the manufacturer's protocol. Isolated total RNA was quantified and assessed for purity using a NanoDrop (Thermo Fisher Scientific, Waltham, MA) and the RNA integrity was confirmed by gel electrophoresis. 1 μg of each total RNA sample was incubated using the ezDNase™ enzyme (Invitrogen, Waltham, MA) to digest any potential gDNA and then reverse transcribed into cDNA using SuperScript™ IV VILO™ (SSIV VILO) Master Mix (Invitrogen, Waltham, MA). Final cDNA products were diluted tenfold in RNAse free water and stored at −20 °C before use.

2.5. Sciaenops ocellatus cDNA cloning and sequencing

Multiple sequence alignments using Clustal Omega (Madeira et al., 2019) were applied. Four or 5 sequences were aligned, with 1 corresponding to another member of the Sciaenidae family, the yellow croaker (*Larimichthys crocea*), and the remaining 3–4 corresponding to other teleost species. Subsequently, primers were designed from consensus sequences representing regions of high homology to amplify large segments of each cDNA of interest in red drum, from which species-specific primers for qPCR could be designed (with the exception of red drum *ef1a*, which was previously published; Esbaugh et al., 2016). Partial cDNA sequences and subsequent target amplicons were cloned using the TOPO TA Cloning Kit (Invitrogen) according to the manufacture's protocol and sequenced by GENEWIZ (South Plainfield, NJ, USA). The specific list of genes sequenced with their respective role during pronephros development and function are presented in Table 1.

Table 1
Table of transcripts involved in pronephric development and function.

Gene name	Gene Symbol	GenBank #	Pronephros importance	References
Vascular endothelial growth factor	*Vegf*	ON098145	Transcription factor expressed by the podocytes in the glomerulus; involved in the formation of the capillary tuft.	*Drummond (2005)*
Vascular endothelial growth factor receptor	*Vegfr*	ON098149	VEGF receptor expressed by endothelial cells involved in the formation of the capillary tuft.	*Drummond (2005)*
Wilms tumor 1 a	*Wt1a*	ON098147	Transcription factor involved in the formation of the podocytes in the glomerulus.	*Perner et al. (2007)*
Podocin	*Podocin*	ON098146	Structural protein involved in podocytes structure and slit-diaphragm formation in the glomerulus.	*Kramer-Zucker et al. (2005)*
Solute carrier family 20 member 1a	*Slc20a1a*	ON098144	Pronephric sodium-phosphate co-transporter expressed by the tubules.	*McCampbell and Wingert (2014)*
Simple-minded 1	*Sim-1*	ON098148	Transcription factor involved in pronephric duct cell specification.	*Serluca and Fishman (2001)*

2.6. qPCR analysis

qPCR was performed using an Agilent AriaMx Real-time PCR System instrument (Agilent, Santa Clara, CA) and Power SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA). Reactions were performed in 96 well plates using a 20 μL final volume: 6 μL sterile water, 10 μL SYBR Green, 1 μL of each forward and reverse primer (to final concentrations of 0.5 μmol L^{-1} each) and 2 μL of diluted cDNA. Settings were as follows: hot start at 95 °C for 3 min, followed by 40 amplification cycles at 95 °C for 5 s and 60 °C for 10 s. Specificity of each primer pair was initially verified by gel electrophoresis and sequencing and subsequently confirmed for each reaction using a terminal melting curve analysis. Primers used are listed in Table S2. Each plate contained all replicates for each time point and WAF treatments and were run in duplicate. The PCR Miner software (Zhao and Fernald, 2005) was used to analyze the raw data and obtain Ct values and reaction efficiencies (E) for each individual reaction. These values were then used to calculate initial fluorescence values (R_0) according to the equation: $R_0 = (1 + E)^{Ct}$. Mean R_0 values were then used to normalize all target gene mRNA expression levels to the expression of the housekeeping gene (*ef1α*). Normalized R_0 values were then expressed relative to the lowest expressed mean value across all time points and treatments (Zhao and Fernald, 2005).

2.7. Whole-mount immunohistochemistry (WM-IHC)

The WM-IHC procedure was performed to observe pronephros morphology and was adapted from previously published methods (Incardona et al., 2004; Bonatesta et al., 2022). Briefly, 14 dpf red drum larvae were randomly collected from control seawater and 1 % HEWAF solutions. Larvae were placed in fixative (20 % DMSO/80 % MeOH) for 2 h at RT. Samples were then treated in 10 % H_2O_2 in fixative for 2 days

to remove potential pigmentation. Samples were then stored in 100 % MeOH at −20 °C until use. Larvae were washed in a graded series of MeOH/PBS and permeabilized for 1 h in blocking solution (PBS, TritonX-100 0.2 %, DMSO 1 %, goat serum 5 %) at RT. Samples were subsequently incubated in monoclonal antibody α6F hybridoma supernatant (DSPH, University of Iowa, Iowa City, IA) (antigen = ATPase, Na + K+ alpha-1 subunit) diluted 1:10 in Blocking solution overnight at 4 °C with gentle shaking. This antibody was previously validated for use in red drum by Allmon and Esbaugh (2017). Larvae were then washed for 3 × 1 h in PBS/0.2 % TritonX-100 at RT, and incubated at 4 °C with the secondary antibody, AlexaFluor488-conjugated goat anti-mouse IgG (α6F) (Molecular Probes, Eugene, OR) diluted 1:1000 in Blocking Solution. This was followed by 3 × 1 h washes in PBS/0.2 % TritonX-100 at RT. Finally, larvae were mounted on concave depression cavity glass microscope slides in 50 % glycerol/50 % PBS for microscope analysis. Pictures of the larvae were captured by using a Zeiss Axiovert 200 M optical microscope (Zeiss, Oberkochen, Germany) with a Yokogawa CSU10 spinning disk confocal attachment (Yokogawa, Tokyo, Japan).

2.8. Statistical analysis

Statistical analyses were performed using SigmaPlot 12.3 (Systat Software, Inc., San Jose, CA). Differences in mRNA expression from the second HEWAF exposure were assessed by two-way ANOVA followed by Holm-Sidak post hoc test (comparison between control and 1 % HEWAF at 36, 60 and 84 hpf). If normality or equal variance failed, data transformation was performed. Specifically, *wt1a* and *slc20a1a* data were log-transformed. No adequate transformation was found for *sim-1* data and a one-way ANOVA at each time point followed by Holm-Sidak post hoc test was performed instead (ANOVA on Rank was performed at 36 hpf). Differences in mRNA expression between control and 1 % HEWAF in 14 dpf larvae were assessed by Student's *t*-test. A one-way ANOVA followed by a Holm-Sidak post hoc test or ANOVA on Rank (when conditions were not met) were performed to analyze differences in survival percentage at each salinity (Control vs HEWAF exposed) per each time point. In all cases, differences were considered significant at $P < 0.05$.

3. Results

3.1. PAHs and water quality

PAH analysis for the first exposure revealed the initial 1 % HEWAF dilution had a concentration of 1.8 μg/L ΣPAH (Table S1). No PAH analysis was performed for the second exposure (data related to mRNA expression analysis) due to an unexpected sampling error. The corresponding data is therefore expressed with the nominal concentration (1 % HEWAF). Specifically, this is applicable to data obtained from qPCR analysis of 36, 60 and 84 hpf larvae (not 14 dpf larvae). Similar 1 % HEWAF preparations using the same crude oil, equipment, preparation method and performed by the same laboratory resulted in 1.8 μg/L ΣPAH (Khursigara et al., 2017). Therefore, a predicted concentration for the second exposure can be reasonably expected to lie in the range of ~1–3 μg/L ΣPAH. Water parameters are depicted in Table S3 for the osmotic challenge test and Table S4 for the 72 h HEWAF exposure test.

3.2. mRNA expression

No statistically significant differences were observed at any time point related to the analyzed transcripts (36, 60 and 84 hpf) for the second HEWAF exposure (Fig. 1). A significant increase in *slc20a1a*, *vegf* and *vegfr* mRNA expression was observed in 14 dpf larvae (~13 d post exposure) previously exposed to 1 % HEWAF (1.8 μg/L ΣPAH; Fig. 2).

3.3. WH-IHC results

Preliminary results showed high cutaneous staining of ionocytes on 72 hpf red drum larvae in both treatments, which precluded analysis/observance of the pronephros (see example in Fig. S1). Therefore, further analysis of pronephros morphology was not pursued.

3.4. Osmotic challenge survival

There were no statistically significant differences in survival between previously HEWAF-exposed larvae and control larvae at any time point in hyperosmotic waters (30 and 18 ppt, Fig. 3). However, previously HEWAF-exposed larvae demonstrated a significant decrease in survival compared to controls in hypoosmotic waters of 4 and 2 ppt. While this decrease was only significant at 72 h after transfer into the 4 ppt treatment, survival was significantly decreased at all time points in the 2 ppt treatment (Fig. 3). Anecdotally, there also appeared to be a high incidence of previously HEWAF-exposed larvae floating on the water surface in the 4 and 2 ppt water treatments (number of individuals not counted). Morphologically, these fish exhibited largely inflated swim bladders (Fig. S2).

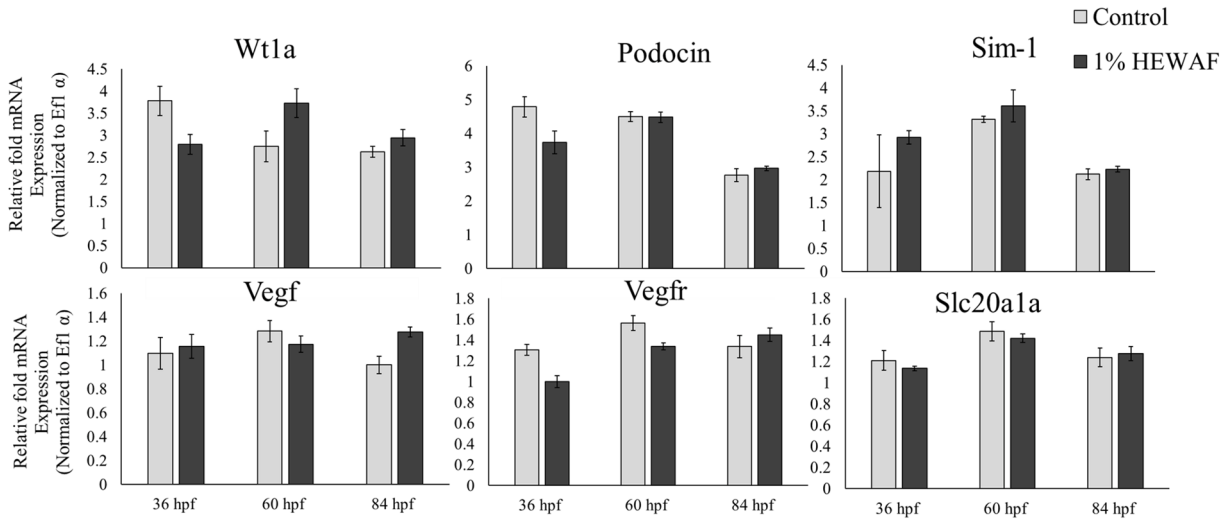

Fig. 1. Relative whole-body mRNA expression levels of *wtla, podocin, sim-1, vegf, vegfr* and *slc20a1a* which are transcripts involved in the development of the pronephros. Transcripts were analyzed in red drum larvae at 24, 48, and 72 h HEWAF exposure (exposure started at 12 hpf). n = 4 (20–40 embryos/larvae each) per timepoint per treatment, run in duplicate. Error bars represent ± SEM. Differences were considered significant at $P < 0.05$.

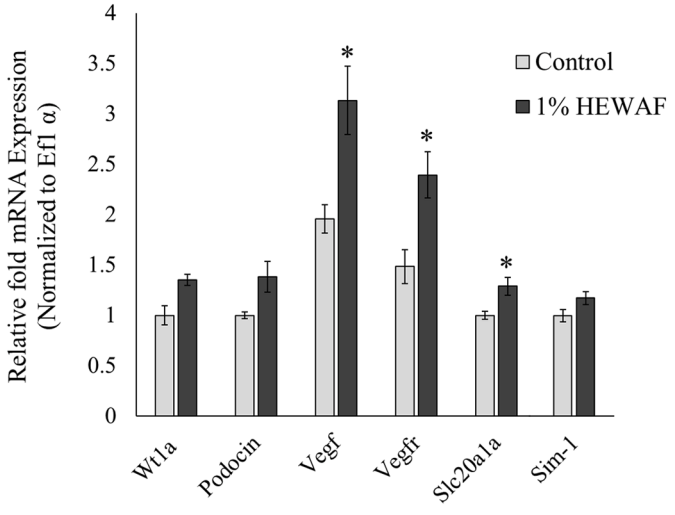

Fig. 2. Relative whole-body mRNA expression levels of transcripts analyzed in 14 dpf red drum larvae following the 24 h HEWAF exposure (exposure started at 12 hpf and ended at 36 hpf). $n = 5$ (10 embryos/larvae each) per treatment, run in duplicate. 1 % HEWAF was measured as 1.8 μg/L ΣPAH, Error bars represent ± SEM. Differences were considered significant at $P < 0.05$ (* represents statistical difference).

4. Discussion

Early-life stage exposure to DWH crude oil is believed to induce development defects in the kidney of teleost fishes (Incardona et al., 2004; Bonatesta et al., 2022). However, it is unknown if these developmental defects result in acute and/or latent functional effects. Kidney developmental defects can have repercussions on the osmoregulatory capability of the organism, and can lead to mortality (Pérez-Rius et al., 2019). This study demonstrated the latent effects of crude oil exposure on the ability of red drum larvae to survive in hypoosmotic waters. Furthermore, mRNA expression of transcripts involved in pronephros development and function were altered in HEWAF-exposed red drum, indicating that an impaired kidney could be contributing to the latent mortality observed following transfer to hypoosmotic waters. Importantly, the measured ΣPAH concentration (1.8 μg/L ΣPAH) and the hypoosmotic conditions (4 and 2 ppt) that red drum larvae experienced during this study are well within the concentrations measured during the DWH spill (coastal measurement as low as 22 μg/L ΣPAH; Whitehead et al., 2012) and expected salinities during the release of riverine water (<5 ppt; McDonald et al., 2015), emphasizing the environmental relevance of the results and suggesting that the release of freshwater may have increased mortality in this species.

A recent study demonstrated that zebrafish larvae exposed to OFS HEWAF (150 μg/L ΣPAH) from ~2 to 96 hpf presented altered expression of selected transcripts involved in pronephros development and function (Bonatesta et al., 2022). Specifically, transcripts were selected based on their role and location within the pronephros, which include the glomerulus (filtration apparatus of the kidney), pronephric tubules and ducts. Transcripts that were altered during the zebrafish study were selected for the current study (role of transcripts summarized in Table 1) to determine whether the observed molecular effects also occur in an environmentally relevant estuarine species at an environmentally relevant ΣPAH concentration. The zebrafish represents a good animal model to identify and analyze potential toxic mechanisms (Drummond, 2000), especially in regard to early kidney development (Drummond, 2005), for which much is known regarding this species. However, due to its relatively low sensitivity to crude oil and PAH toxicity (Incardona, 2017; Grosell and Pasparakis, 2021) and the fact that it is a freshwater species, the zebrafish is not an environmentally relevant species regarding the DWH oil spill. Furthermore, the ΣPAH concentration tested in the previously mentioned study (150 μg/L ΣPAH; Bonatesta et al., 2022) fall in the higher range of ΣPAH concentrations measured in the GoM during the spill (open ocean: 189 μg/L ΣPAH; coastal area: > 200 μg/L ΣPAH; Diercks et al., 2010; Whitehead et al., 2012; respectively). On the other hand, a very low ΣPAH concentration was quantified during our test (1.8 μg/L ΣPAH; Table S1).

Notably, no differences in expression of all analyzed transcripts were observed during the 72 h static HEWAF exposure (from 12 to 84 hpf; Fig. 1), implying that the observed molecular responses in ELS zebrafish were not observed in red drum larvae. This could be due to a less severe

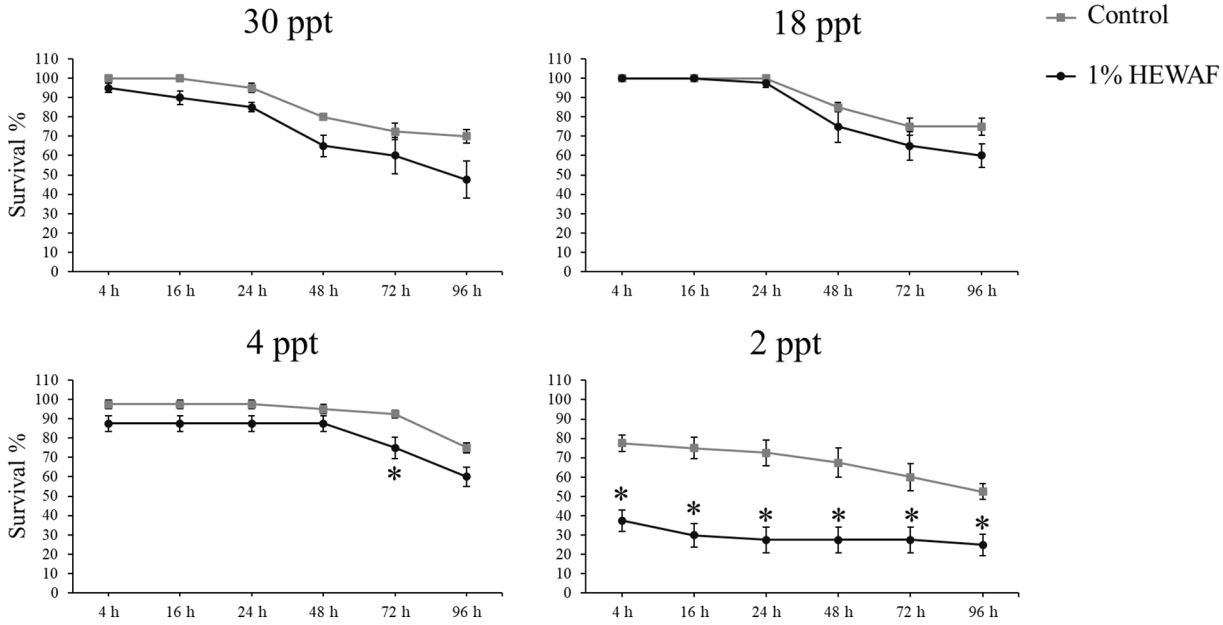

Fig. 3. 96 h red drum osmotic challenge test survival result. Red drum larvae were exposed to control seawater or 1.83 μg/L of Σ50 PAH (1 % HEWAF) for 24 h from 12 to 36 hpf and then transferred into sterilized seawater up until 14 dpf. Larvae were then transferred into waters with various salinities (2, 4, 18 and 30 ppt) for 96 h and percentage survival was assed at different times ($n = 4$, 10 larvae per replicate). Differences were considered significant at $P < 0.05$ (* represents statistical difference).

toxic insult induced by the lower ΣPAH concentration used or differences in species-specific responses. Alternatively, the kidney of red drum might develop at a slower pace compared to that of a typical freshwater fish, as red drum are spawned in offshore marine waters and generally develop the capacity to osmoregulate in hypoosmotic waters during the weeks prior to estuarine nursery recruitment (Holt et al., 1981). This is in contrast to zebrafish which exhibit a functional pronephros as early as 40 to 48 hpf (Drummond, 2003). Therefore, the lack of similar mRNA expression responses between these two species may simply reflect different timing and/or rates of kidney development and their corresponding developmental sensitivities to HEWAF exposure. However, mRNA expression of *slc20a1a*, *vegf* and *vegfr* were significantly altered in 14 dpf red drum larvae that were previously exposed to DWH crude oil for 24 h (from 12 to 36 hpf; Fig. 2). Interestingly, an increase in *slc20a1a* mRNA expression was observed in red drum larvae. *Slc20a1a* is solely expressed in the pronephric tubules and is associated with a sodium-dependent phosphate transporter (McCampbell and Wingert, 2014; Bonatesta et al., 2022). Therefore, this observation could represent a latent osmoregulatory response adopted by the tubules to compensate for a potential alteration of internal osmotic homeostasis. Increased expression of s*lc20a1a* was also observed in zebrafish larvae after a 96 h exposed to 75 μg/L ΣPAH only, but not at higher concentrations (Bonatesta et al., 2022).

A significant increase in *vegf* and *vegfr* mRNA expression was observed in 14 dpf red drum larvae that were previously exposed to 1 % HEWAF. *Vegf* mRNA expression was also observed in zebrafish larvae previously exposed to 150 μg/L ΣPAH for 96 h, but only after 3 d recovery in clean water (Bonatesta et al., 2022). Although the zebrafish exposure is not directly comparable to our study, it is interesting to observe altered expression of these transcripts days after the exposure and not during or immediately after the exposure in both studies. Fish recover from renal injury by neonephrogenesis (Reimschuessel, 2001; McCampbell and Wingert, 2014); therefore, it is possible that the altered mRNA expression of *vegf* and *vegfr* is likely a compensatory response related to the regenerative capabilities of the kidney by fishes. It is important to mention that these transcripts are not solely involved in pronephros development and can play roles in other mechanisms and pathways (Bonatesta et al., 2022). For example, increased mRNA expression of *vegf* and *vegfr* has been observed during cyst formation, which could be a symptom of nephrotoxicity (Tao et al., 2007), as well as during hypoxic signaling pathway activation (Hendon et al., 2008; Scholz and Kirschner, 2011). Therefore, cyst formation as a result of crude oil-induced nephrotoxicity, hypoxic signaling activation as a result of crude oil-induced cardiotoxicity and the ensuing reduction in systemic oxygen supply should be considered as plausible contributing factors. Overall, the results indicate that some latent molecular responses continue well after HEWAF exposure has ceased in red drum ELSs, potentially as compensatory or regenerative mechanisms to cope with the initial insult to the pronephros or, alternatively, as potential indicators of cyst formation and nephrotoxicity.

Given that the kidney plays a crucial role in osmoregulation (McDonald, 2007), any defects associated with its development can result in acute and/or latent osmoregulatory defects. As hypothesized, red drum larvae that were previously exposed to 1 % HEWAF from ~12 to 36 hpf and reared in clean seawater up until 14 dpf, were more sensitive to hypoosmotic waters (4 and 2 ppt waters) compared to control larvae (Fig. 3). Specifically, HEWAF exposed larvae demonstrated a significant decrease in survival throughout the entire test duration (96 h) in 2 ppt, and after 72 h in 4 ppt. Interestingly, a decrease in survival was not observed in exposed red drum larvae compared to control when challenged in hyperosmotic waters (30 and 18 ppt). As previously mentioned, in hypoosmotic waters fish need to compensate for the water gain and ion loss, and do so by excreting a large volume of water through diluted urine and by actively absorbing ions (Marshall and Grosell, 2005). In hyperosmotic waters, fish need to compensate for the water loss and ion gain, and do so by actively absorbing water (by the gastrointestinal tract and the kidney) and actively excreting ions across the gills and through isotonic urine (Marshall and Grosell, 2005). The fact that the survival effects of crude oil exposure were most dramatic in 2 ppt water is perhaps not surprising considering that it represents a more extreme departure from the internal osmolarity of the fish when compared to the 30 ppt salinity (i.e., 5-fold vs. 3-fold difference from internal osmolarity of ~10 ppt), and from the salinity that they were raised in (35 ppt).

The developing kidney plays an important role in water excretion relatively early in fishes (Hentschel et al., 2007; Fedorova et al., 2008; Rider et al., 2012). Early renal clearance capacity is observed in other estuarian fish species, such as the turbot (*Scophthalmus maximus*) and the herring (*Clupea harengus*) after just a few days post-hatch (Tytler et al., 1996). Tytler and Ireland (2000) demonstrated an early ability of herring larvae to regulate urine flow rate (UFR) at various salinities. Remarkably, a 2.5× increase in UFR was observed in 11–14 dph larvae at 4 ppt salinity compared to the 34 ppt salinity control as a response to the hypoosmotic environment exposure (Tytler and Ireland, 2000). If water excretion through the pronephros is reduced or inhibited, it might result in water accumulation within the fish larvae, which can be observed by the presence of edema (Kramer-Zucker et al., 2005; Perner et al., 2007). In fact, it was recently shown that zebrafish larvae previously exposed to DWH crude oil presented an enlarged edema in a concentration-dependent fashion, likely due to a reduced ability to excrete water. This effect was significantly exacerbated when the pre-exposed larvae where challenged in hypoosmotic waters for 24 h, whereas it was reduced/negated when challenged in waters close to isosmotic conditions (Bonatesta et al., 2022).

Aside from the earlier zebrafish study (Bonatesta et al., 2022), to our knowledge there are no studies that have examined the direct effect of crude oil exposure on the ELS teleost kidney development and osmoregulatory function. Looking at single PAH exposure, Incardona et al. (2004) have shown that zebrafish larvae exposed to phenanthrene, a 3-ringed PAH found in crude oil, presented pronephric morphological defects, characterized by straightening of the pronephric tubules and the presence of cysts. It was not possible to observe these morphological defects during the current study due to the inadequacy of the technique used to observe the pronephros in red drum (Fig. S1). Outside of kidney osmoregulatory function, there is a recent study that has examined DWH crude oil effects on the kidney of adult Gulf toadfish (*Opsanus beta*) associated to hormonal regulation by the hypothalamic–pituitary–interrenal axis (Cartolano et al., 2021). Notably, Cartolano et al. (2021) also showed that the isolated kidney of toadfish exposed to DWH crude oil (2.8 μg/L ΣPAH) for 7 days presented an increased mRNA expression of cytochrome P4501A1, which is known to be a biomarker for the activation of the aryl hydrocarbon receptor by PAHs exposure (Billiard et al., 2002). Furthermore, there are a couple of studies that have examined the effect of crude oil exposure in the mammalian kidney (Adedara et al., 2012; Ramesh et al., 2018). Specific to DWH crude oil, adult male mice exposed intraperitoneally to crude oil or a mixture of crude oil and Corexit (dispersant) solutions showed impairment of kidney function, characterized by increased concentration of creatinine and urea nitrogen in the blood (Ramesh et al., 2018). Altogether, the above-mentioned studies and the data obtained from red drum in the current study suggest that the reduced red drum survival in hypoosmotic waters was likely driven, at least in part, by persistent crude oil-induced nephrotoxicity.

Early life stage fishes are characterized by having a thin skin and a high surface area to volume ratio (Burggren et al., 2017), which are ideal conditions for epidermal exchanges with the external environment. Therefore, before other regulatory organs are fully developed and/or functional, the epidermis is vital in osmoregulation (Varsamos et al., 2005; Fridman, 2020).Throughout growth and development, the integument tends to grow thicker and the surface area to volume ration grows smaller (Burggren et al., 2017; Kwan et al., 2019). Consequently, new organs are needed to fulfil the regulatory functions initially

achieved by the epidermis; therefore, other biological structures (e.g., pronephros, gills) advance from a rudimentary stage to a more advanced stage, and assume their functional osmoregulatory roles (Drummond, 2003; Varsamos et al., 2005; Kwan et al., 2019; Fridman, 2020). However, little information is known about their specific roles during fish ELSs. This lack of information is mostly due to the logistical constraints associated with analyzing such functions in small organisms. Based on ELS zebrafish, the onset of pronephric function appears to precede that of other osmoregulatory organs, such as the gills (Rombough, 2002). Given the scarcity of information related to the development of osmoregulatory strategies in red drum larvae, we cannot exclude that other tissues and/or mechanisms have been affected by the early crude oil exposure. In fact, PAH and crude oil exposure has been shown to induce histological changes in the gills (Kennedy and Farrell, 2005; Katsumiti et al., 2008), as well as potentially directly influence ion uptake pathways (Larsen et al., 2014). Yet, the high survival in hyperosmotic conditions suggests that general osmoregulatory pathways related to ion excretion and water absorption are functional. Instead, the fast timeline (<4 h of exposure) for the reduced survival in the hypoosmotic 2 ppt water highly suggests that the issue is related to impaired water excretion and ion uptake/retention.

This study demonstrates the high sensitivity of DWH crude oil exposed red drum larvae to hypoosmotic waters in the salinity range reported within the GoM estuaries during the spill. Importantly, the exposure to the toxicant occurred around 2 weeks prior to the osmotic challenge, demonstrating a latent effect of crude oil exposure. A similar latency in effect was also previously observed for ocular function following a transient embryonic exposure of red drum to crude oil (Magnuson et al., 2018). Furthermore, the measured ΣPAH concentration during our study was 1.8 μg/L ΣPAH which is in the lower range of the measured concentration during the spill. In fact, measured ΣPAH concentrations in Barataria Bay (where riverine water was released; (Baker et al., 2017) were around 200 μg/L ΣPAH in May 2010 and around 22 μg/L ΣPAH by the end of June 2010 (Whitehead et al., 2012). Consequently, the observed effects during this study could have been more severe in the natural environment, and the released riverine water around the cost of the GoM could have exacerbated the mortality of larval red drum (Rose et al., 2014). Furthermore, the majority of the pre-exposed larvae that survived the 96 h osmotic challenge at 2 and 4 ppt displayed a highly inflated swim bladder which led the larvae to float on top of the test water surface with the inability to properly swim. Although not measurable as direct mortality, this could contribute to reduced fitness and alter foraging ability and predator-prey interactions in the natural environment. The results from this study should help inform future oil spill response efforts, particularly in terms of regulating freshwater flows to mitigate the effects of spill threatened estuarine environments.

5. Conclusion

Early life stage DWH crude oil exposure in red drum induced latent osmoregulatory defects. This was particularly noticeable in red drum larvae challenged in hypoosmotic waters, especially at 2 ppt, which represented the most extreme osmotic challenge. Given the important osmoregulatory role that the teleost kidney plays in hypoosmotic freshwaters and that ELS crude oil exposure induces kidney developmental defects, it was hypothesized that defective renal function was associated with the observed mortality. As evidence, red drum larvae exposed to a very low HEWAF dilution during our study presented altered latent expression of transcripts involved in kidney development. Furthermore, a previous study in which the transcription of these same genes was altered demonstrated that DWH crude oil exposed zebrafish larvae showed reduce/inhibited renal water clearance (Bonatesta et al., 2022). Since the ΣPAH concentrations applied during our test is below the concentrations that have been quantified during the DWH spill, it is possible that more severe outcomes could have been experienced in the natural environment, emphasizing the environmental relevance of our study for future risk assessments and managements.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2022.109405.

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