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ASSESSMENT OF EARLY LIFE STAGE MAHI-MAHI WINDOWS OF SENSITIVITY DURING ACUTE EXPOSURES TO *DEEPWATER HORIZON* CRUDE OIL

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Abstract: Windows of exposure to a weathered *Deepwater Horizon* oil sample (slick A) were examined for early life stage mahi-mahi (*Coryphaena hippurus*) to determine whether there are developmental periods of enhanced sensitivity during the course of a standard 96-h bioassay. Survival was assessed at 96 h following oil exposures ranging from 2 h to 96 h and targeting 3 general periods of development, namely the prehatch phase, the period surrounding hatch, and the posthatch phase. In addition, 3 different oil preparations were used: high- and low-energy water accommodated fractions of oil and very thin surface slicks of oil (~1 μm). The latter 2 were used to distinguish between effects due to direct contact with the slick itself and the water underlying the slick. Considering the data from all 3 exposure regimes, it was determined that the period near or including hatch was likely the most sensitive. Furthermore, toxicity was not enhanced by direct contact with slick oil. These findings are environmentally relevant given that the concentrations of polycyclic aromatic hydrocarbons eliciting mortality from exposures during the sensitive periods of development were below or near concentrations measured during the active spill phase. *Environ Toxicol Chem* 2017;36:1887–1895. © 2017 SETAC

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INTRODUCTION

The 2010 *Deepwater Horizon* oil spill culminated in the release of millions of barrels of crude oil into the northern Gulf of Mexico [1]. Importantly, the spill overlapped in time and space with the spawning of resident pelagic fish species, including mahi-mahi (*Coryphaena hippurus*), yellowfin tuna (*Thunnus albacares*), and many others [2–9]. Measurements summarized in the 2016 Final Programmatic Damage Assessment and Restoration Plan [10] place concentrations of polycyclic aromatic hydrocarbons (PAHs) in the upper subsurface waters during the spill ranging as high as 240 $\mu g\,L^{-1}$. Recently, the acute toxicity thresholds were characterized for early life stage mahi-mahi exposed to various preparations of *Deepwater Horizon* crude oil, revealing a high level of sensitivity that varied depending on the state of weathering, with 96-h median lethal concentrations (LC50s) ranging from 8.8 $\mu g\,L^{-1}$ Σ50 PAHs (highly weathered) to 45.8 (nonweathered) $\mu g\,L^{-1}$ Σ50 PAHs [11]. Although the 96-h exposure period used in that study [11] followed a standard bioassay approach designed to assess mortality over the putatively most sensitive initial stages of life, it is likely that some oil exposures in the environment were more transient in nature (e.g., 24 h or less). Moreover, such exposures may have transpired during different periods of development that vary in sensitivity within the 96-h time frame. Yet, it remains unclear whether a shorter duration exposure to oil at an environmentally relevant concentration, occurring during the particularly sensitive period(s) of development, is sufficient to elicit acute mortality.

Pelagic teleosts inhabiting the warm surface waters of the Gulf of Mexico undergo rapid development during the initial embryonic phase, thus rendering these fish highly susceptible to sustaining developmental effects from relatively short exposures to contaminants. Work over the past 10 yr to 15 yr has identified the cardiovascular system in particular as a primary target of PAH toxicity in developing early life stage fish [12–22]. Indeed, recent reports have shown that early life stage mahi-mahi and other large pelagic fish exhibit a suite of functional and morphological cardiovascular defects within a 48-h exposure to very low concentrations of *Deepwater Horizon* oil (~1 to 20 $\mu g\,L^{-1}$ ΣPAHs) [11,23–25]. Such effects on the developing heart are believed to contribute to, if not outright cause, the oil-induced mortality observed during an acute bioassay. In support of this, pericardial edema, one of the hallmark traits of cardiotoxicity, was recently shown to be correlated with acute lethality of early life stage mahi-mahi following 96-h exposure to *Deepwater Horizon* oil, although reduced atrial contractility was not [11]. Studies of herring and zebrafish have revealed that the cardiotoxic syndrome arises as a result of functional deficiencies in conduction of the newly-beating heart that hinders proper late-stage cardiac morphogenesis (e.g., cardiac looping) [12–15]. Recent evidence suggests the underlying mechanism of this functional impairment is largely through blockade of potassium and calcium channels involved in the excitation-contraction coupling mechanism of cardiomyocytes [26]. In addition, recent time-dependent transcriptional analyses of early life stage mahi-mahi have linked oil-induced molecular responses to the cardiotoxic phenotype as well as revealed novel targets

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aside from the heart likely involved in the developmental toxicity of *Deepwater Horizon* oil [23,27]. All told, there is mounting evidence that exposure to PAHs during a transient window of sensitivity critical to development (cardiac or otherwise) could impart imminent lethality. Examining the windows of sensitivity within the course of a 96-h bioassay may lend important insight regarding the potential mode(s) of action underlying such toxicity.

In addition to a rapid early life stage development, another feature common among some pelagic teleosts in the Gulf of Mexico is a broadcast spawning strategy that produces embryos that are positively buoyant until the hours proximal to hatch (~36 h postfertilization [hpf] for mahi-mahi). At least 43 300 square miles (112 115 km^2) of the northern Gulf of Mexico were cumulatively covered by oil slicks of varying thickness as a result of the *Deepwater Horizon* spill [10]. Once formed, these slicks may serve as a source of PAH release into the underlying water, producing a gradient of PAH concentrations that decreases with depth. Thus, the developing embryos of mahi-mahi in close proximity to oil slicks during the spill presumably would have experienced greater exposure to PAHs and hence greater toxicity. Furthermore, there is the potential for added toxicity because of direct contact with the slick itself, either through enhanced exposure to PAHs within the slick or through other unforeseen factors associated with the microenvironment or physical coating of the embryo with oil (e.g., reduced oxygen availability). Although novel insights into the physical contributions of crude oil toxicity have been gained from previous studies of zebrafish and pink salmon, indicating that the primary role of particulate oil in embryonic fish toxicity is that of a reservoir for dissolved PAH release [28,29], still little is known regarding the influence of surface slicks in exerting toxicity during crude oil exposures to the developing embryos of pelagic broadcast spawners in the northern Gulf of Mexico.

In light of this information, the main objectives of the present study were to characterize the primary window(s) of sensitivity during 96-h exposures of early life stage mahi-mahi to *Deepwater Horizon* crude oil prepared as 1) a high-energy water accommodated fraction (HEWAF), 2) a thin surface slick, and 3) a low-energy water accommodated fraction (LEWAF). The LEWAF method produces total PAH concentrations and compositions in the water column similar to that using the slick method without producing a surface slick, thereby aiding in distinguishing effects attributable to the surface slick itself and those attributable to the dissolved PAHs in the underlying water. Median lethal effect concentrations were determined from the HEWAF and LEWAF exposures using a series of 96-h bioassays with varying windows of oil exposure ranging from 2 h to 96 h in length. A similar set of experiments was performed for the slick exposures; however, because LC50s could not be calculated since only a single slick dosing method (i.e., thickness) was used, test performance was instead reported as 96-h percentage of survival. The findings reported herein should aid in garnering a more comprehensive understanding of the underlying factors that influence crude oil toxicity to early life stage pelagic fish as well as help inform future risk and injury assessment efforts.

MATERIALS AND METHODS

Experimental animals

Mahi-mahi broodstock were captured off the coast of Miami, Florida, USA, in an area free of any known contamination, using hook and line angling techniques. The fish were then transferred to the University of Miami Experimental Hatchery and acclimated in 80 m^3 fiberglass maturation tanks (typically 5–7 per tank) equipped with recirculating aquaculture systems for water quality and temperature control. All embryos used in the experiments described in the present study were collected within approximately 2 h to 8 h following a volitional (non-induced) spawn using standard methods [30]. A prophylactic formalin (37 mg L^{-1}) treatment was administered to the embryos, followed by a 0.5-h rinse with a minimum of 300% water exchange by volume. All tests were initiated within 0.5 h to 1.5 h following rinse. A small sample of eggs was collected from each spawn to assess fertilization rate and embryo quality via microscopy. Spawns with low fertilization rates (<85%) or frequent morphological abnormalities (>5%) were not used. All handling and use of animals in the present study were in compliance with the guidelines of the Institutional Animal Care and Use Committee of the University of Miami.

Experimental design

Three types of exposure regimes were employed to examine windows of sensitivity for early life stage mahi-mahi over the 96-h period typically used for standard acute toxicity tests, which for mahi-mahi encompasses the period from newly spawned embryos up to when first larval feeding would occur. The first regime utilized exposures to HEWAF preparations of crude oil performed in custom-made pelagic embryo-larval exposure chambers (PELECs; described in the section *Toxicity testing*) [31]. The second regime utilized a method to produce thin surface slicks in beakers [32,33] to examine acute mortality attributable to direct contact of embryos with slick oil during the positively buoyant early stages of development. The third regime utilized LEWAF preparations in beakers [34,35] to help distinguish between the effects of direct slick contact and the effects associated with dissolution of the slick oil into the underlying water. Exposure windows were selected to examine sensitivity during the predominantly prehatch embryonic stage, periods entirely spanning hatch, and the predominantly posthatch larval stage. The qualifying term "predominantly" is used because embryos become negatively buoyant and settle to the bottom of the vessel in the approximately 2 h to 4 h just proximal to hatch, which typically begins at approximately 36 h; however, there is variability among the exact timing of hatch within a given cohort. Thus, for exposure periods starting -or ending at approximately 36 h, there was typically a mixture of floating embryos, settled embryos, and hatched larvae, although in all cases most were settled embryos.

Preparation of WAFs and surface slicks

The oil used in the present study (referred to as slick A) was collected during the *Deepwater Horizon* spill on 29 July 2010 from the hold of barge number CTC02404, which was receiving slick oil from various skimmer vessels (sample ID CTC02404-02), and was subsequently transferred under chain of custody to the University of Miami. All WAFs were prepared in accordance with standardized methods established for the *Deepwater Horizon* Natural Resource Damage Assessment toxicity testing program [36]. Each HEWAF was prepared on the day of use by mixing 1 g oil/L seawater using a Waring CB15 blender at low speed for 30 s. The mixture was immediately transferred to a glass separatory funnel and allowed to settle for 1 h, and then the lower 90% was collected and used for exposures. Low-energy water accommodated fractions were prepared the day before use in 5-L aspirator bottles at a loading rate of 2 g oil/L seawater and were mixed at low speed (no vortex) using a stir plate for 18 h to 24 h. After

mixing, the lower 90% was collected and used for exposures. Surface slicks were generated in 1 L of seawater in 1-L glass beakers by evenly spreading 2 g of oil along the entire inner circumference (~1 cm wide band) of a 3-inch diameter polyvinyl chloride (PVC) pipe and suspending the band of oil beneath the water surface for 4 h [32,33]. This method generates very thin surface sheens of approximately 1 μm in thickness [32]. All of the water used in the experiments described herein was 1 μm-filtered, ultra violet (UV)-sterilized seawater.

Toxicity testing

In total, 29 tests (6 HEWAF, 16 slick, and 7 LEWAF) were performed employing various windows of oil exposure within a 96-h period (Figure 1). Four replicates were used per treatment in all tests. Each WAF test consisted of 5 WAF dilutions and a control (Supplemental Data, Table S1). Water accommodated fraction dilutions were generated as bulk solutions by vigorously mixing appropriate volumes of WAF and seawater using a stir plate and then aliquoted into replicate exposure vessels. Because of the relatively low maximum PAH concentration of the LEWAF preparation, all LEWAF tests used the same treatment dilutions, each decreasing by 2-fold from a maximum 100% treatment, except for the control. Once all exposure vessels were prepared with treatment water or slick, freshly-fertilized embryos were promptly collected under a dissecting microscope and added to each vessel well beneath the water surface using a wide-bore glass pipette. Fish were not fed during the course of the exposures.

For the HEWAF exposures, each replicate consisted of 40 embryos in 1.8 L of test solution held in a PELEC [31]. Briefly, this recirculating exposure system consisted of a 1-L glass Imhoff cone customized with a Teflon stopcock on the bottom and an overflow spout at the top for draining into a 1-L glass beaker. Overflow was recirculated through silicone tubing from the beaker back to the cone via the stopcock using a peristaltic pump at a flow rate of 90 mL min^{-1} to 110 mL min^{-1}. Fish were retained in the cone using a glass excluder extending from the overflow drain with nylon mesh fastened on both sides with silicone O-rings. For the LEWAF and slick exposures, each replicate consisted of 20 embryos in 1 L of test solution held in a glass beaker.

Use of the PELEC for the HEWAF tests, which were performed first, was initially favored over using static 1-L beaker exposures because the PELEC offered 3 advantages: 1) greater control survival, as previously demonstrated for mahi-mahi and yellowfin tuna [23,31], increased the likelihood of test success; 2) the system allowed for test solution exchanges (e.g., adding or washing out WAF) without direct handling of the fish or modifying the test volume; and 3) the larger overall volume and upwelling nature of the exposure system allowed for a greater number of fish used per replicate vessel (40 vs 20), thereby increasing statistical power. However, the PELEC also presented a drawback in that the greater surface area, recirculation, and use of silicone and nylon components all facilitated a more rapid and greater overall depletion of PAHs over time. This precluded use of the PELECs for the LEWAF tests, which have inherently low maximum starting concentrations as a result of their low-energy preparations. This was confirmed by a preliminary 96-h LEWAF trial with the PELECs that failed to elicit any discernable mortality even at the 100% dose (18.9 μg L^{-1} and 0.1 μg L^{-1} Σ50 PAHs for initial and final concentrations, respectively; remaining data not shown; see Supplemental Data, Figure S1 for PAHs included in Σ50 calculation). Thus, all subsequent tests (LEWAF and slick) were performed using 1-L beakers, given the much slower rate of PAH depletion associated with this method.

Low-energy water accommodated fraction and slick tests were monitored daily for survival. The nature of the exposure vessel for the HEWAF tests prevented an accurate daily count of live and dead animals before test take-down; therefore, survival was scored only at test termination (96 h). This was accomplished by first draining the vessel through the bottom stopcock into a 1-L beaker in which live and dead animals could be accurately counted. Mortality was assessed visually without the aid of a microscope and by lack of response to prodding. All dead animals were removed from each test daily using glass pipettes (except for the HEWAF tests). Survival tests were deemed unreliable if control survival at hatch was less than 70%

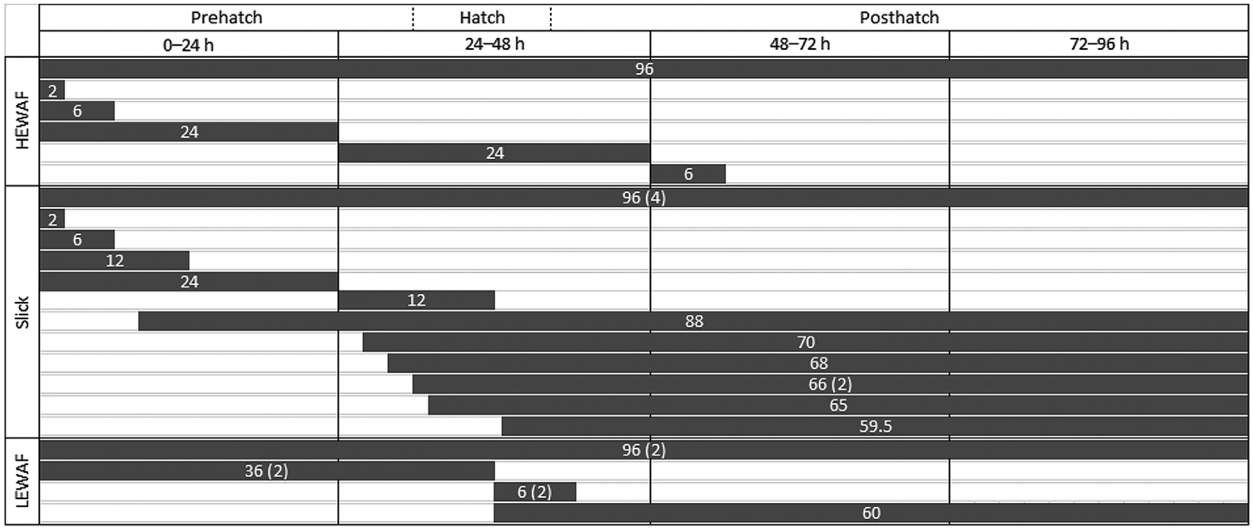

Figure 1. Schematic illustration of the windows of exposure for each of the high-energy and low-energy water accommodated fractions (HEWAF/LEWAF) of oil and surface slicks tests reported in the present study. Shaded areas indicate periods of oil exposure during a 96-h test. Values inside shaded areas indicate duration of exposure (h); parenthetical values indicate the number of times a test was performed if >1. Note that the period of hatch is variable, occurring between approximately 30 h and 40 h, although it typically begins at approximately 36 h. All tests were initiated approximately 4 h to 10 h postfertilization.

or if the subsequent survival of hatched larvae at 96 h was less than 80% (i.e., minimum of 80% survival of 70% hatched, or 56%). The defined hatch point for the purposes of test reliability was 48 h, as mahi-mahi typically hatch between 30 hpf to 40 hpf when raised under ambient conditions (27 °C). Because hatch rate could not be accurately assessed for the HEWAF tests, these tests were deemed unreliable if survival of control larvae at 96 h was less than the initial 70% cut-off for survival at hatch used for the beaker tests. All tests were performed in a temperature-controlled environmental chamber (27 °C) with a 16:8-h light: dark cycle.

Methods for achieving windows of exposure

Initiating HEWAF exposures in the PELECs at a time other than time 0 was accomplished by briefly stopping flow, closing the stopcock, replacing the 1 L of overflow water in the beaker with a 1.8× concentrated WAF dilution, and quickly returning flow. This approach facilitated a gradual attainment of a final 1× WAF dilution in the cone containing the fish after sufficient time had passed to reach equilibrium with the beaker solution (~1 h). For terminating a WAF exposure at a time other than 96 h, a similar procedure was followed except that clean seawater was pumped through the cone via the stopcock and drained to a waste container via the outflow spout for sufficient time to achieve near complete washout (0.5 h). In addition, the water in the beaker (wiped clean with a paper towel) was replaced with clean seawater before returning the system to recirculating flow. Composite samples (10 mL total) were collected from each set of replicates 1 h after washout to confirm near complete WAF removal by fluorescence analysis of total PAH concentration [11,37]. Typical washout was >98% and was always >96%. All test solution exchanges were also performed for controls using clean seawater.

Windows of exposure for LEWAF and slick tests were accomplished by preparing a second set of 1-L beakers containing 1 L of control seawater, LEWAF dilution, or slick-overlaid seawater into which embryos or larvae were transferred at the appropriate time using a wide bore glass pipette. For the slick exposures, embryos were added as gently as possible to minimize the temporary disruption of the slick. For terminating exposures to LEWAF or slick oil for durations shorter than 96 h, embryos or larvae were returned in the same manner to the original corresponding clean seawater beaker, where they were then held and monitored until the end of the 96-h period. Both transfer and no-transfer controls were included in the initial tests; however, because statistical analysis revealed that the transfer process did not affect survival (Kruskal-Wallis one-way analysis of variance [ANOVA] on Ranks; $p = 0.216$), the transfer controls were typically omitted from later tests. Unlike the HEWAF tests that were difficult to monitor for the period of hatch, this period was easily determined in the 1-L beakers used for the slick and LEWAF tests. This was accomplished by monitoring the beakers for hatch at 25-min to 35-min intervals beginning approximately 26 h after test initiation, an approach that allowed for the finer resolution exposures beginning or ending near hatch for these tests.

Water quality and PAH analysis

Polycyclic aromatic hydrocarbon samples were collected in 250-mL amber bottles and shipped overnight on ice to ALS Environmental (Kelso, WA, USA) for analysis by gas chromatography/mass spectrometry–selective ion monitoring (GC/MS-SIM; based on US Environmental Protection Agency [USEPA] method 8270D [38]). Reported ΣPAH values represent the sum of 50 PAH analytes (23 parent + 27 alkyl homologs) selected for standard analysis for the *Deepwater Horizon* Natural Resource Damage Assessment toxicity testing program (Supplemental Data, Figure S1) [36]. For the HEWAF tests, initial samples from exposures initiated at time 0 were collected from bulk dilutions, whereas initial samples from all other exposures were collected as composites from the outflow spout of replicate exposure vessels after 1 h of addition to allow for complete mixing; final samples were also collected as replicate composites. For the LEWAF tests, samples for PAH analysis were collected either directly from bulk dilutions (initial) or collected as composites from replicate beakers (final). For the slick tests, samples were collected as replicate composites by siphoning beneath the slick layer using tubing connected to a glass pipette that was fastened to the inner side of the beaker prior to slick formation. The pipette was positioned so that the opening was approximately midway between the surface and bottom. Approximately 50 mL to 100 mL of the samples were allowed to pass for tube priming before actual sample collection.

For all experiments, temperature, pH, dissolved oxygen, and salinity were measured at least once daily, and total ammonia was measured at the conclusion of each test. Temperature and dissolved oxygen were measured using a ProODO handheld optical dissolved oxygen probe and meter (YSI) and pH was measured using a Radiometer PHM201 meter fitted with a combination glass electrode. The pH and dissolved oxygen probes were calibrated daily. Salinity was measured using a refractometer and total ammonia was determined using a colorimetric assay [39]. Summaries for all measured water quality parameters, ΣPAH concentrations, and test survival rates are provided in Supplemental Data, Table S1.

Statistical analyses

To calculate 96-h LC50s, exposure–response curves were fit using the USEPA's TRAP Ver 1.21A software. Data from each test were fit to a 2-parameter tolerance type Gaussian model using log-transformed exposure concentrations (Supplementary Data, Figures S2 & S3). The LC50s were calculated for Σ50 and Σ3-ring PAHs using only the initial concentrations or the geometric mean of initial and final concentrations (corresponding to the window of exposure) [40]. Polycyclic aromatic hydrocarbons (Σ3-ring) represented the 18 tricyclic PAHs included within the Σ50 PAHs (Supplemental Data, Figure S1). In some cases, values of 0 were obtained for measured ΣPAH concentrations (i.e., all analytes were below detection limits). Because logarithmic transformation and geometric means were used in calculating LC50s, neither of which can accommodate values of 0, all measured PAH concentrations with values of 0 were substituted for one half the lowest detection limit among the individual PAHs included in the respective ΣPAHs. These 0-substituted values corresponded to $0.0022\ \mu g\ L^{-1}$ and $0.0068\ \mu g\ L^{-1}$ for Σ50 and Σ3-ring PAHs, respectively. For the slick tests, statistical comparisons were made among the following groups using Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's multiple comparison method: controls, predominantly prehatch, spans prehatch and post-hatch, and predominantly posthatch. Differences were tested for statistical significance using SigmaStat Ver 3.5 (Systat Software) and were deemed significant at $p < 0.05$. Comparisons of LC50s were based on overlapping 95% confidence

intervals, with nonoverlapping intervals signifying a significant difference between estimates.

RESULTS

PAH concentration and composition

As expected, nominal dilutions of HEWAF and LEWAF produced nearly proportional changes in initial PAH concentrations (Supplemental Data, Table S1 & Figure S4). Regardless of the oil preparation method (HEWAF, LEWAF, or slick), the 3-ring PAHs (mainly fluorenes, dibenzothiophenes, and phenanthrenes/anthracenes) comprised the greatest relative percentage of Σ50 PAHs, representing approximately 60% (Supplemental Data, Figure S1). The remaining approximately 40% of PAHs in the slick and LEWAFs, which had similar overall PAH profiles, were mainly comprised of 2-ring PAHs (~36%). However, the relative percentages of 2-ring and 4-ring PAHs decreased and increased to approximately 20%, respectively, in the HEWAFs. Common to each oil preparation method was an enrichment of 3-ring and 4-ring PAHs over time due to the greater loss of 2-ring PAHs, likely from volatilization (Supplemental Data, Figure S4). Notably, however, the PAH concentrations decreased more rapidly and to a greater extent in the HEWAF exposures, likely due in large part to the nature of the PELEC system and removal of droplitized oil in the HEWAF from the water column due to adsorption to surfaces and coalescing and rising to the water surface. High-energy water accommodated fraction Σ50 PAH concentrations decreased on average by 28%, 55%, and 92% after 2 h, 6 h, and 24 h, respectively (Supplemental Data, Table S1). By contrast, Σ50 PAH concentrations for the exposures performed in beakers decreased on average by 69% after 96 h in the LEWAF exposures and by approximately 50% from 2 h to 96 h in the slick exposures. Polycyclic aromatic hydrocarbon concentrations rapidly increased in the slick exposures from 0 h to 2 h and then decreased over time thereafter (Supplemental Data, Figure S4E). However, only 1 time 0 sample was collected from a mock exposure beaker (as opposed to composites of 4 collected for the other time points) and this value may simply reflect an atypically low concentration due to random variation. Once formed, the slick typically remained visible on the surface for at least 24 h.

HEWAF exposures

Six HEWAF bioassays were performed: 1 full 96-h exposure test and 5 others designed to examine periods of sensitivity in the hours preceding, spanning, and following hatch (Figure 2). Final samples for the 96-h exposure bioassay were mistakenly not collected and therefore geometric mean LC50s could not be directly calculated for this test. However, by estimating final concentrations of PAHs using 5% of the corresponding initial concentrations it was possible to calculate an approximate geometric mean LC50. This 95% depletion of PAHs after 96 h was selected as a conservative estimate based on the 92% depletion on average observed after 24 h (Supplemental Data, Table S1). Using this approach, it is clear that the nearly complete depletion of PAHs by 24 h effectively limited test exposures to this duration as evident by the nearly identical LC50s for the 0-h to 24-h and 0-h to 96-h tests (Figure 2). Comparing prehatch exposure durations of 2 h, 6 h, and 24 h initiated at the start of each test revealed that toxicity increased with increased duration of exposure out to 24 h. However, the following 24-h window (24–48 h), which spans the entire period of hatch (typically ~36 h), exhibited the greatest sensitivity to

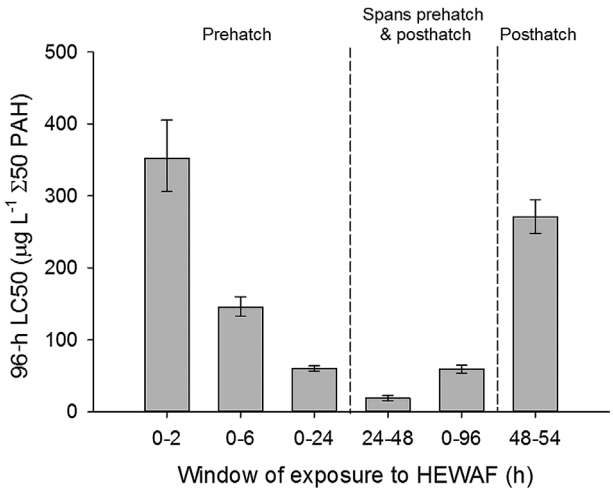

Figure 2. Assessment of early life stage mahi-mahi windows of sensitivity to slick A high-energy water accommodated fraction (HEWAF) exposure using the pelagic embryo-larval exposure chamber exposure system. Median lethal concentrations (LC50s) of 96 h were calculated following various windows of exposure within a 96-h test period using the geometric mean of initial and final Σ50 polycyclic aromatic hydrocarbon concentrations (corresponding to the exposure period). Exposure periods either preceded, spanned, or followed the period of hatch (typically ~36 h) as indicated. Error bars represent 95% confidence intervals. Note that the 0-h to 96-h LC50 was approximated using a final concentration estimated as 5% of the initial concentration.

HEWAF exposure. Comparing 6-h exposure windows during the early embryonic stage (0–6 h) versus the early larval stage (48–54 h) revealed a greater sensitivity during the former. Similar trends as those just described were also observed when comparing LC50s calculated using only Σ3-ring PAHs (Supplemental Data, Table S2).

Slick exposures

Final Σ50 PAH concentrations across all slick tests were relatively consistent despite different exposure durations, with an overall mean ± standard error of the mean (SEM) value of $2.4 \pm 0.3\ \mu g\ L^{-1}$ (Supplemental Data, Table S1). Mean control survival across all slick tests was 83%, whereas mean survival following 96 h of slick exposure was 36% (Figure 3). Percentage of survival was similar to controls if the slick exposure occurred prior to hatch; however, if the slick exposure spanned the hatch period or occurred during the predominantly posthatch larval period, survival was significantly reduced compared to controls or with the prehatch exposure group. It should be noted for the 2 posthatch tests that, despite the different exposure start times, the exposures were initiated at approximately the same developmental stage (mostly settled embryos proximal to hatch). The 31-h to 96-h test was performed at a different time of year (September vs April/May for all other slick tests) and the difference in hatch time was likely related to slight variations in maturation tank water temperatures and spawning times that naturally occur over time.

LEWAF exposures

Four different exposure periods were examined for the LEWAF tests (Figure 1). With the exception of the 36-h to 96-h exposure period, tests were performed twice for each period. Data from repeated tests were used both individually and combined to generate LC50s (Figure 4; Supplemental Data, Table S2). Regardless of how the data were analyzed, no clearly discernible window of higher sensitivity was found when

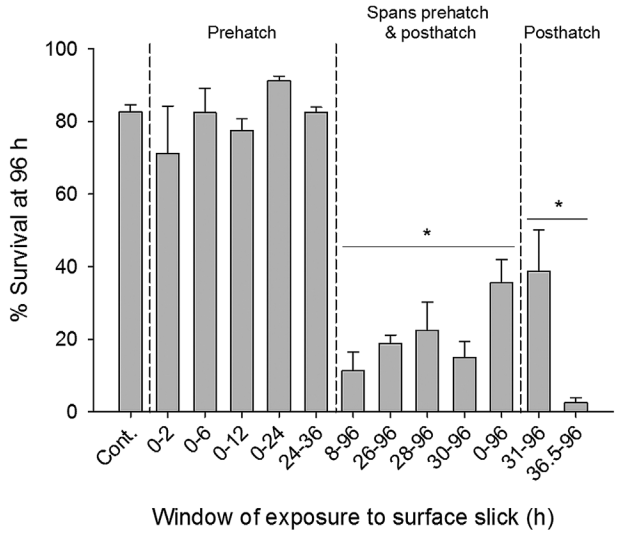

Figure 3. Assessment of early life stage mahi-mahi windows of sensitivity to surface slick exposure using slick A. Exposure windows targeted various prehatch stages (predominantly prehatch for the 24–36-h test), periods encompassing hatch, or the predominantly posthatch larval stage as indicated. Note that for the latter exposures, most of the fish were negatively buoyant embryos at the initiation of exposure and therefore would not have experienced any direct contact with the slick. Note also that for the controls, the 0-h to 96-h period and the 30-h to 96-h period, mean values were calculated from multiple tests (51, 16, and 8 total replicates from 7, 4, and 2 tests, respectively; controls include transfer and no-transfer controls). Data are represented as mean ± standard error of the mean. *Indicates significant difference from controls and the prehatch group by Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's multiple comparison procedure.

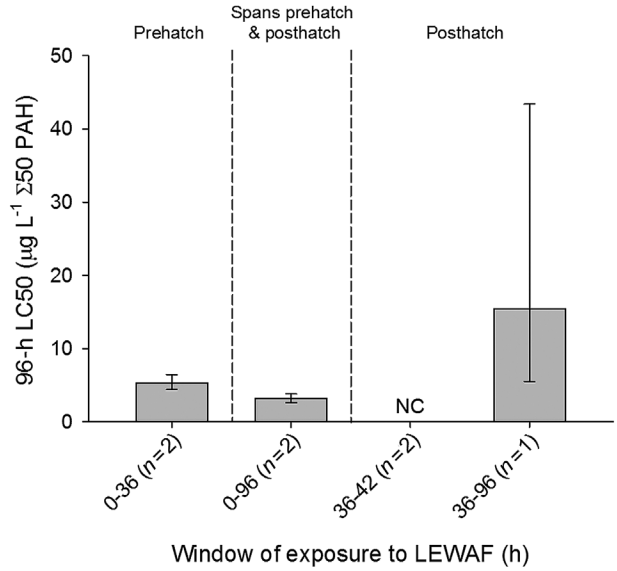

Figure 4. Assessment of early life stage mahi-mahi windows of sensitivity to slick A low-energy water accommodated fraction (LEWAF) exposure. Median lethal concentrations (LC50s) at 96 h were calculated following various windows of exposure within a 96-h test period using the geometric mean of initial and final Σ50 polycyclic aromatic hydrocarbon concentrations (corresponding to the exposure period). Exposure windows targeted the predominantly prehatch embryonic stage or the predominantly posthatch larval stage as indicated. Note that except for the 36-h to 96-h test, each test was performed twice. Data from repeated tests were grouped and regressed together to generate a combined LC50 (see Supplemental Data, Table S2 for individual test LC50s) except for the 36-h to 42-h tests, for which LC50s could not be calculated (NC) due to insufficient mortality. Error bars represent 95% confidence intervals.

comparing the predominantly prehatch (0–36 h) to the predominantly posthatch (36–96 h) period. Furthermore, the mortality observed from a full 96-h exposure could not be fully attributed to an exposure spanning either of these periods when considering LC50s using combined data. An LC50 for the 36-h to 42-h exposure period could not be calculated because of insufficient mortality. Similar trends were also observed when comparing LC50s calculated using only Σ3-ring PAHs (Supplemental Data, Table S2).

DISCUSSION

The present study sought to determine whether there are discernible windows of sensitivity to *Deepwater Horizon* crude oil exposure during the 96-h period of a standard early life stage toxicity test for mahi-mahi. Specifically, focus was placed on 3 general periods of development, namely the prehatch stage, the late embryonic/early larval stage spanning hatch, and the posthatch larval stage. Three different types of highly-weathered (slick A) oil preparations were employed: HEWAF, surface slick, and LEWAF. The HEWAF method was chosen because of the high level of sensitivity to slick A (96-h LC50 of 8.8 [7.4–10.3] $\mu g\,L^{-1}$ Σ50 PAHs) utilizing this method previously demonstrated for early life stage mahi-mahi using static exposures in 1-L glass beakers [11]. In the present study, initial tests utilized slick A HEWAF preparations using the custom recirculating PELEC system [31]. Although this approach was initially favored over using static 1-L beakers for reasons described previously (see *Materials and Methods*), the PELEC also presented a drawback due to more rapid and greater overall depletion of PAHs over time. In fact, the average loss of Σ50 PAHs after 24 h was 92%, thus effectively limiting the duration of a representative exposure in the PELEC to approximately 24 h, as evidenced by the nearly identical LC50s obtained from the 0-h to 24-h and 0-h 96-h exposures. This accelerated loss of PAHs also explains the large discrepancy in 96-h LC50s obtained in the present study ($252\,\mu g\,L^{-1}$ Σ50 PAHs) and that found previously using 1-L static beaker exposures ($8.8\,\mu g\,L^{-1}$ Σ50 PAHs; comparison represents LC50s calculated using initial concentrations because finals were not measured in the previous study) [11]. Clearly, the former LC50 reflects the higher concentrations required in the dilution series to induce mortality from a much shorter effective exposure (∼24 h) in the PELEC.

Although the faster rate of PAH depletion with the PELEC system precluded the ability to make direct comparisons of 96-h LC50s with the previous static beaker test, windows of sensitivity could nevertheless still be assessed with the PELEC system, although the windows were naturally confined to 24 h or less. Of the 4 possible nonoverlapping 24-h windows to examine, only the first 2 were tested in entirety in the present study. The third 24-h window (48–72 h) was examined only over the first 6 h, and no test was performed specifically targeting the final 24-h period (72–96 h). Focus was directed toward dissecting the first 48 h of exposure because previous work has shown that exposure during this period is highly sensitive and critical for developing the cardiotoxic phenotype [11,14,23,24,41]. Although certainly not definitive, the observation of a higher LC50 for the 48-h to 54-h test compared with the 0-h to 6-h test tends to support the posthatch larval period as being less sensitive. Comparing the first two 24-h exposure periods, the second period was over 2-fold more sensitive based on geometric mean LC50s. This likely indicates that the period surrounding or including hatch is the most sensitive period of development for mahi-mahi exposed to

crude oil, although additional studies will be needed to determine whether there is a narrower window of sensitivity that defines the toxicity observed within this period. Nevertheless, it is interesting to note that significant mortality resulted from exposure during any of the 3 periods examined (prehatch, spanning hatch, or posthatch) despite the developmental differences associated with each (discussed below). In any event, these findings are significant considering that the Σ50 PAH LC50s of 19 $\mu g\,L^{-1}$ (24–48 h) and 146 $\mu g\,L^{-1}$ (0–6 h) are below some concentrations measured in the Gulf of Mexico during the active spill phase [10,42–44], suggesting that exposures to *Deepwater Horizon* crude oil as transient as 6 h to 24 h may have caused significant mortality to early life stage mahi-mahi and likely other large pelagic fish with similar sensitivities [24].

The second (slick) and third (LEWAF) exposure regimes were designed to distinguish between effects associated with direct exposure to a thin surface slick of oil (~1 μm) and the predominantly dissolved components of oil beneath the slick. Mahi-mahi embryos are initially positively buoyant and thus float to the surface or near surface where they reside until the hours proximal to hatch (~36 h). At such time, the embryos become negatively buoyant and sink to lower depths that are presumably more beneficial for survival as developing larvae [45]. Thus, it is highly likely that the developing embryos of mahi-mahi were in close proximity or directly exposed to slicks during the spill that would have presumably resulted in greater exposure to oil and hence greater toxicity. Although transient exposures to slick oil were clearly acutely toxic, eliciting as much as 97% mortality, surprisingly this toxicity could not be attributed to exposure occurring solely during the predominantly buoyant prehatch phase. By contrast, exposures that either included or were initiated near the hatching phase, when most of the embryos were negatively buoyant, elicited significant toxicity (~80% mortality on average). These results suggest that physical contact with thin films (~1 μm) of slick oil over varying windows during the 36 h proximal to hatch is not acutely toxic to mahi-mahi, but rather acute toxicity arises from exposure to the underlying water during the hours near or after hatch. Although the depth to which mahi-mahi embryos sink prior to hatching is unknown, field-collected Σ50 PAH concentrations have been reported as high as 240 $\mu g\,L^{-1}$ in the upper 20 m of the subsurface waters [10], indicating potential exposures during the *Deepwater Horizon* spill well above the LEWAF LC50s reported in the present study during embryo descent and likely during and after hatch.

Results from the LEWAF exposures tend to support that exposure to PAHs in the underlying water is the primary factor eliciting toxicity; however, the sensitive period of exposure was less clear from these tests. No clearly discernible window of higher sensitivity to LEWAF was found when comparing the prehatch (0–36 h) to the posthatch (36–96 h) periods. This presents an apparent contrast with the results from the slick and HEWAF tests, which clearly revealed periods including or initiated near hatch as more sensitive. A possible explanation is that the period surrounding hatch is indeed the period most sensitive to PAH exposure and that any apparent discrepancies with this observation reflect natural variation and lack of resolution in the timing of hatch. Although 36 h was often chosen as the time to end or initiate an exposure to oil, in an attempt to more closely examine the periods leading up to and following hatch, it was impossible to control for the precise timing of hatch; however, in such tests most of the larvae were unhatched and settled to the bottom of the beaker at the time of transfer (and thus likely near to hatch). Moreover, there is growing evidence that critical windows of sensitivity are not defined by strict cut-off points but rather are more plastic and better represented as a continuum of increasing and decreasing sensitivity [46]. Thus, any exposure extending near the 36-h time point may have resulted in greater or lesser exposure during the critical window of sensitivity surrounding hatch depending on natural variability in hatching time or plasticity of the window itself. This may help explain, for example, the apparent differences in sensitivity exhibited by exposures leading up to the 36-h time point for the LEWAF (0–36 h) and slick (24–36 h) tests. Lack of resolution around the timing of hatch could also help explain the absence of a clear distinction in sensitivity in the periods preceding or following hatch for the LEWAF exposures.

Focusing further on the period around hatch, there is developmental and physiological evidence in support of this period as representing the most sensitive. It has become clear over the past 2 decades that the most conspicuous form of injury in early life stage teleosts exposed to crude oil is a cardiotoxic syndrome characterized predominantly by pericardial and yolk sac edema. Studies of zebrafish have demonstrated that the syndrome likely arises due to exposure coinciding with the period approximately 12 h after onset of the heart beat (~24 hpf in zebrafish) and during formation of the atrioventricular conduction pathway which disrupts heart function and subsequent late-stage cardiac morphogenesis [14,15]. Onset of the heartbeat in mahi-mahi also begins at approximately 24 hpf [6; P. Perrichon, University of North Texas, Denton, TX, USA, personal communication] and the cardiac syndrome is clearly present in oil-exposed mahi-mahi [11,41] and other large predatory pelagic fish [24] by approximately 48 h (~12 h following hatch). These findings are therefore consistent with those of zebrafish indicating that the period approximately 12 h after onset of the heartbeat is a critical window of sensitivity which also coincides with the period near hatch in mahi-mahi (~36 h). Intriguingly, recent work has also shown that metabolic rate rises steeply in mahi-mahi near the period of hatch [47]. Thus, a diversion of energy utilization toward PAH metabolism during this critical period of high metabolic demand coinciding with hatch may also contribute to the high level of sensitivity observed during this period. Taken together, the weight of evidence from the present study and previous studies tends to support the period surrounding hatch as likely the most sensitive.

It is also clear, however, that mortality can result from exposures during less sensitive periods either well before or well after the period around hatch in mahi-mahi. The observation that significant, albeit sometimes different, levels of mortality arise from exposures occurring within different developmental periods (i.e., prehatch, posthatch, or period surrounding hatch) raises some interesting considerations regarding bioaccumulation of PAHs and potential mode(s) of toxic action. For example, a 6-h HEWAF exposure either shortly after fertilization or approximately 12 h following hatch was sufficient to induce significant mortality at 96 h. Considering the 0-h to 6-h exposure, lipophilic PAHs are likely to concentrate in the lipid-rich yolk sac during embryonic exposures and may serve as a toxicant sink from which PAHs are mobilized at later periods of development as the yolk sac is depleted [48,49]. Thus, internal PAH exposures could arise during the key period of atrioventricular conduction pathway formation (~36 h) due to mobilization of stored PAHs from the yolk sac accumulated during the 0-h to 6-h exposure. Indeed, recent work has shown

that mahi-mahi embryos exposed to weathered *Deepwater Horizon* crude oil deplete their yolks at a faster rate than controls, thus potentially facilitating exposure via this route [47]. Future studies combining an assessment of PAH bioaccumulation/depuration rates with a time course of traditional cardiac morphometrics should help clarify or confirm the toxic mode(s) of action in mahi-mahi transiently exposed to crude oil during these stages as well as the role, if any, that yolk-liberated PAHs might play in this process. With respect to exposures occurring solely during the posthatch larval stage, the absence of a chorion and greater surface area to volume ratio no doubt contribute to a more rapid bioaccumulation of PAHs [48]. Considering the previously established effects of tricyclic PAHs on the developing teleost heart [12–22], the observation of similar trends among LC50s calculated using Σ50 and Σ3-ring PAHs tends to support a role for cardiotoxicity contributing to mortality across all time points and oil preparations examined; however, the extent of this contribution from exposures initiated during the larval stage remains largely unknown at this time given that such exposures occurred after the critical period of atrioventricular conduction pathway formation. A recent time course RNA-Seq study has implicated additional less overtly-apparent impacts that may also contribute to the developmental toxicity of *Deepwater Horizon* oil to mahi-mahi, specifically impaired vision, cell viability, metabolism, neural development, and steroid biosynthesis [27]. Yet, these novel molecular responses will need to be phenotypically anchored to higher levels of biological organization in the future to more firmly link their roles in mediating crude oil toxicity as well as contributing to potential windows of sensitivity.

In conclusion, the present study revealed that exposures to environmentally relevant concentrations of PAHs of much shorter duration than 96 h can impart significant toxicity to mahi-mahi. Importantly, although the period surrounding hatch was identified as likely the most sensitive to crude oil exposure for mahi-mahi, toxicity was also elicited by exposures during other developmental periods, the timing of which may shed additional light on potential toxic mode(s) of action. In addition, it is clear that exposure to thin surface slicks of oil are acutely toxic to mahi-mahi, although likely as the result of exposure to leached PAHs into the underlying water rather than a direct contact effect. In sum, these findings provide a more comprehensive understanding of the mode, timing, and duration of crude oil exposure that elicit acute mortality of early life stage mahi-mahi and therefore should aid in evaluating the effects imparted by the *Deepwater Horizon* spill to mahi-mahi and other early life stage pelagic fish native to the Gulf of Mexico. Furthermore, considering the conserved phenotypic responses of early life stage fish following exposure to oils from varying geological sources [15], these findings should also aid risk and injury assessment efforts for future oil spills impacting sensitive pelagic species.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3713.

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Data Availability—Data may be accessed by contacting the authors directly (emager@unt.edu).

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