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Parental exposure to Deepwater Horizon oil in different environmental scenarios alters development of sheepshead minnow (*Cyprinodon variegatus*) offspring

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ABSTRACT

The explosion of the Deepwater Horizon (DWH) oil exploration platform on April 20, 2010 began a catastrophic leak of approximately 640 million liters crude oil into the northern Gulf of Mexico (GOM), affecting more than 2100 km of coastline, including wetlands and estuaries that provide habitat and nursery for many aquatic species. Estuaries of the GOM are dynamic environments, with constant fluctuations in salinity and dissolved oxygen, including large hypoxic zones during summer months. Spawning fish in northern GOM estuaries following the DWH incident were at significant risk of oil exposure, and adverse environmental conditions at the time of exposure, such as hypoxia and low salinity, could have exacerbated developmental effects in the offspring. The present study investigated the effects of F_0 parental oil exposure in different environmental scenarios on development of F_1 sheepshead minnow (SHM) offspring. Adult SHM were exposed to the high-energy water accommodated fraction (HEWAF) of crude oil in three environmental scenarios: normoxic (NORM), hypoxic (HYP), and hypoxic with low salinity (HYP-LS). Parental HEWAF exposure in the NORM scenario resulted in developmental effects in F_1 offspring, including altered heart rate, decreased length at hatch, and impaired prey capture. Co-exposure of F_0 SHM to HEWAF and adverse environmental conditions altered HEWAF effects on F_1 heart rate, hatch rate, prey capture, and survival. Time to hatch was not significantly impacted by parental HEWAF in any environmental scenario. The present study demonstrates that parental exposure to HEWAF results in developmental changes in F_1 embryos, and co-exposure to adverse environmental conditions altered the effects for several developmental endpoints. These data suggest that SHM exposed to oil in estuaries experiencing hypoxia or low salinity may produce offspring with worsened outcomes. These developmental effects, in addition to previously reported reproductive effects in adult fish, could lead to long-term population level impacts for SHM.

1. Introduction

The explosion of the Deepwater Horizon (DWH) oil exploration platform on April 20, 2010 began a catastrophic leak of approximately 640 million liters of crude oil into the northern Gulf of Mexico (GOM), creating an oil slick that covered more than 112,000 km^2 of the ocean's surface (Beyer et al., 2016; McNutt et al., 2012). The spill also affected more than 2100 km of coastline, including wetlands and estuaries that provide habitat and nursery for many aquatic species (Beyer et al., 2016; Nixon et al., 2016). Estuaries of the GOM are dynamic environments, with constant fluctuations in salinity and dissolved oxygen, including large hypoxic zones during summer months. Salinity is influenced by the flow of fresh water from the Mississippi River, and is typically lowest in the spring when river flow into the Gulf is at its peak (Love et al., 2013). Dissolved oxygen levels of the northern GOM are impacted by excess nutrient runoff from the Mississippi River

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watershed, water stratification, and a seasonal increase in temperature that increases biochemical oxygen demand (Diaz and Rosenberg, 2008; Thomas et al., 2007; USEPA, 2006).

Spawning fish in northern GOM estuaries following the DWH incident were at significant risk of oil exposure. This is of particular concern as early life stages of fish are generally considered to be more sensitive to contaminant exposure than adult fish (Hutchinson et al., 1998; McKim, 1977) and early life exposure to PAHs can negatively impact fitness and survivorship later in life (Brown et al., 2016; Incardona and Scholz, 2016; Mager et al., 2014). Exposure to polycyclic aromatics hydrocarbons (PAHs) can cause numerous developmental effects in fish, including reduced hatch percentages, delayed hatching, reduced length, cardiac abnormalities and increased mortality (Bosker et al., 2017; Brewton et al., 2013; Incardona et al., 2014; Rodgers et al., 2018). These developmental effects are well-documented in model fish species (de Soysa et al., 2012) and in GOM residents (Denslow et al., 2015; Dubansky et al., 2013; Heintz et al., 2000; Incardona et al., 2014; Rodgers et al., 2018), including sheepshead minnow. (Bosker et al., 2017; Denslow et al., 2015; Jasperse et al., 2019b). Sheepshead minnows (*Cyprinodon variegatus*; SHM) are small-bodied (< 8 cm long) euryhaline fish that reside in shallow waters along the east coast of the United States (Bigelow and Schroeder, 1953), including estuaries of the northern GOM. Sheepshead minnows have broad tolerance for wide ranges of environmental conditions (Nordlie, 2006), and are used extensively for toxicology studies, including effluent toxicity testing by the United States Environmental Protection Agency (USEPA, 2002).

While the effects of oil exposure on fish development are well-investigated, most studies expose the embryo or larvae directly (Dubansky et al., 2013; Incardona et al., 2014; Wu et al., 2012). Few studies have investigated the effects of parental (F_0) PAH exposure on development of offspring (F_1). In one study, zebrafish (*Danio rerio*) with chronic dietary exposure to PAHs produced offspring with reduced heart rate, increased larvae size, and behavioral stress (Perrichon et al., 2015). In another study, marine medaka (*Oryzias melastigma*) exposed to phenanthrene generated offspring with reduced time to hatch and increased heart rate (Sun et al., 2015). While these studies provide valuable insight into the impact of F_0 oil exposure on F_1 development, they were performed using species not relevant to the GOM, which may not predict the effects in GOM residents. Moreover, residents of GOM estuaries may have been exposed to oil while experiencing adverse environmental conditions common to the northern GOM, such as hypoxia and low salinity, which could have exacerbated developmental effects in their offspring. Importantly, recently published data demonstrated that hypoxia and low salinity exacerbated the adverse effects of high-energy water-accommodated fraction (HEWAF) exposure on SHM reproduction (Jasperse et al., 2019a). The present study, therefore, investigated the effects of F_0 HEWAF exposure in different environmental scenarios on development of F_1 SHM to determine if suboptimal environmental conditions exacerbate the effect of oil on development of offspring.

2. Methods

2.1. Sheepshead minnow

Sheepshead minnow were obtained from the University of Southern Mississippi (USM) and bred to establish a colony at the University of Connecticut. All fish housing protocols and experimental procedures were approved under University of Connecticut's Institutional Animal Care and Use Committee protocol A15-059.

2.2. F_0 sheepshead minnow high-energy water-accommodated fraction (HEWAF) exposure

Adult F_0 SHM (> 120 dph) were exposed to high-energy water-accommodated fraction (HEWAF) under three different environmental scenarios as previously described (Jasperse et al., 2019a). Sheepshead minnow were placed in 20 L glass tanks, with 3 females and 2 males per tank. During reproductive experiments, adult SHM were fed flaked food (Zeigler Aquatox Fish Diet; Zeigler Bros, Inc., Gardners, PA) twice a day and freshly hatched brine shrimp (*Artemia salina*) nauplii once a day (Brine Shrimp Direct). Acclimation to the experimental conditions was achieved by gradually changing water parameters over a 10-d period. Sheepshead minnow were exposed to HEWAF continuously for 14 d (n = 6 tanks per treatment), using a flow-through system, as previously described (Jasperse et al., 2019b). A breeding net made of a PVC ring covered with fine nylon mesh (335 μm) was placed into each F_0 tank to provide a spawning substrate and allow collection of embryos for assessment of developmental endpoints. The F_0 HEWAF exposure was performed in three environmental scenarios: normoxic (NORM), hypoxic (HYP), and hypoxia with low salinity (HYP-LS). For the HYP and HYP-LS scenarios, a header tank of seawater was sparged with nitrogen (N_2) to reduce dissolved oxygen levels. Water temperature was maintained at 30 °C for all three scenarios and monitored daily. Dissolved oxygen (DO) levels were monitored daily in a subset of the experimental tanks using YSI 5420 sensors (YSI Incorporated, Yellow Springs, OH) and were 6 mg/L for the NORM scenario and 2.5 mg/L for the HYP and HYP-LS scenarios. Salinity was kept at 15 ppt for NORM and HYP scenarios, and 10 ppt for the HYP-LS scenario, and was monitored using Sybon Opticon Series FG100sa refractometer (Bethesda, MD, USA). The isosmotic point for SHM was reported to be 10.5 ppt (Adeyemi and Klerks, 2012), therefore SHM were hypoosmotic in the NORM and HYP scenarios, and near isosmotic in the HYP-LS scenario. Measured conditions for each scenario are reported in Table S1.

Importantly, the water conditions of all scenarios were within normal ranges for the northern GOM (USEPA, 1999). The somatic and reproductive effects of HEWAF exposure on F_0 SHM under those environmental scenarios are reported in Jasperse et al. (2019a). Fig. 1 illustrates the overall experimental design and outlines the abbreviated names for F_1 groups that will be used throughout the manuscript.

2.3. High-energy water-accommodated fraction (HEWAF) preparation

HEWAF was prepared according to protocols previously described (Incardona et al., 2013; Jasperse et al., 2019b). Briefly, Louisiana sweet crude (LSC) oil (surrogate, SO-20111116-MPDF-003) was mixed with 3 L artificial seawater (15 ppt; Instant Ocean®, Blacksburg, VA, USA) in a Waring CB15 high-speed commercial blender (Torrington, CT, USA) at 1 g oil/L seawater for 1 min on low speed. The HEWAF was prepared in 7 batches (total of 21 L), poured into a 23-L glass carboy, and allowed to settle for 1 h, then collected using a peristaltic pump, avoiding the oil-water interface. The HEWAF was mixed with artificial seawater in header tanks at dilutions (v/v) of 0% (no HEWAF; control), 1.25% (low HEWAF), and 12.5% (high HEWAF) and administered to individual F_0 exposure tanks by passive flow. The HEWAF for the HYP-LS scenario was prepared as described above, but with 10 ppt artificial seawater. The concentrations of individual parent and alkyl PAHs in the HEWAF preparations for each scenario were reported in Jasperse et al. (2019a), and a summary of values are presented in Table 1.

2.4. F_1 generation

Embryos (F_1) were collected from breeding nets on days 7, 10, and 13 of F_0 HEWAF exposure, rinsed, and placed into embryo cups, which consisted of a petri dish attached to a cylinder of fine nylon mesh (335 μm). There was one embryo cup per tank (6 tanks per treatment) for each embryo collection, and a maximum of approximately 50 embryos per embryo cup (Table S2). Embryo cups with F_1 embryos were maintained through 10 days post hatch (dph) in tanks of clean aerated seawater, with static renewal every other day. Seawater was maintained at salinity 15 ppt, temperature 30 °C, and dissolved oxygen 6 mg/L. Importantly, F_1 received no additional exposure to HEWAF,

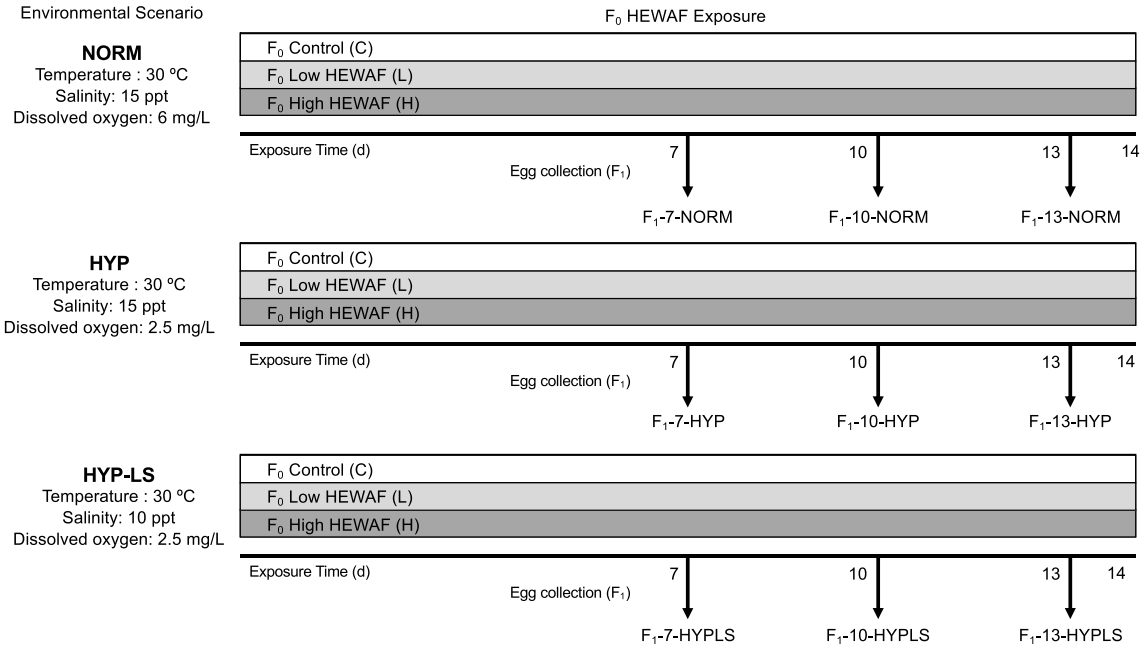

Fig. 1. Experimental design for assessment of F_1 generation development. F_0 sheepshead minnow (SHM) were exposed to high-energy water accommodated fraction (HEWAF) in three related, but independent experiments, in three different environmental scenarios: normoxic (NORM), hypoxic (HYP), or hypoxic with low salinity (HYP-LS). F_0 adult SHM were exposed to 0%, 1.25% (Low) or 12.5% (High) HEWAF for 14 d. Embryos were collected from breeding nets on days 7, 10, and 13 of F_0 HEWAF exposure to assess developmental effects in a F_1 generation raised in clean water (no oil).

Table 1
Composition of stock high-energy water accommodated fraction (HEWAF) prepared in three environmental scenarios: normoxic (NORM), hypoxic (HYP), and hypoxic with low salinity (HYP-LS). All stock HEWAF samples were prepared with a loading rate of 1 g oil/L seawater, in 15 ppt seawater for the NORM and HYP scenarios and 10 ppt seawater for the HYP-LS scenario. Samples were analyzed using gas chromatography-tandem mass spectrometry (GC-MS/MS) and are expressed as mean ± standard error in ng/ml (n = 5 for NORM and HYP, n = 2 for HYP-LS). Concentrations of tPAHs in low and high HEWAF were calculated as 1.25% and 12.5% the measured concentration of stock HEWAF, respectively. PAHs = polycyclic aromatic hydrocarbons.

Total Parent PAHs (ng/ml)	NORM n = 5	HYP n = 5	HYP-LS n = 2
Stock HEWAF	137 ± 13	119 ± 4	84 ± 5
Low HEWAF (1.25%)	1.7	1.5	1.1
High HEWAF (12.5%)	17	15	10.5

hypoxia, or low salinity after embryo collection (< 24 h post fertilization). Larvae were fed freshly hatched brine shrimp nauplii once per day.

2.5. Developmental endpoints

The following developmental endpoints were assessed: heart rate, hatch rate, time to hatch, larval length at hatch (Length0), larval length at 10 dph (Length10), prey capture at 10 dph, and survival to 10 dph. Heart rates were determined 2 days after embryo collection using an inverted microscope (25x magnification) and counting heart beats one time over a 30-s period. Embryos were then returned to embryo cups and monitored daily for hatching to determine hatch rate and time-to-hatch. Once hatched, a subset of larvae, representing all embryo cups, were photographed on a microscope to determine standard length using AxioVision 4.8.1 software (Zeiss, Oberkochen, Germany) and then returned to the embryo cups. Length at hatch could not be measured in embryos that hatched after the earliest day of hatching for each embryo cup, as they could not be distinguished from embryos that had already been photographed and measured. At 10 dph, larvae were counted to determine survival and a subset, representing all embryo cups, were photographed on the microscope to determine standard length. Prey capture was also assessed in the larvae on 10 dph, in a manner similar to methods previously described (Jasperse et al., 2019b; Weis et al., 2003). Larvae were placed individually into a well of a 48-well plate containing five or six brine shrimp in approximately 750 µl seawater. The number of remaining brine shrimp was monitored at 2 and 5 min to determine the ability of larvae to capture prey.

Embryos collected on the 13th day of HEWAF exposure in the NORM scenario (F_1-13-NORM) experienced a heater malfunction and were therefore excluded from analysis of all developmental endpoints.

2.6. Statistical analyses

One-way analyses of variance (ANOVAs) with Holm-Sidak test were used to determine differences between HEWAF exposures and control for each developmental endpoint. Normality was assessed using Kolmogorov–Smirnov test, and equal variance was tested with the Levene median test. A one-way ANOVA on Ranks with Dunn's test was used when data violated normality assumptions. T-test was used when comparisons were made between two groups. All analyses were performed using SigmaStat 3.5 software (Systat Software, San Jose, CA), using an alpha level of 0.05 for statistical significance. All data are presented as mean ± standard error of the mean (SEM) unless indicated otherwise.

Biological replicates were F_0 tanks or individual embryos, depending on the endpoint (*tanks*: hatch rate, time to hatch, survival; *embryos*: heart rate, length0, length10, prey capture). Statistical analyses could not be performed for groups with n < 3 (sample sizes are indicated in figures).

3. Results

3.1. Heart rate

Changes in heart rates in the F_1 embryos produced by fish exposed to HEWAF under the three environmental scenarios are shown in Fig. 2. F_1-7-NORM-L and F_1-7-NORM-H embryos had heart rates 5–8% higher

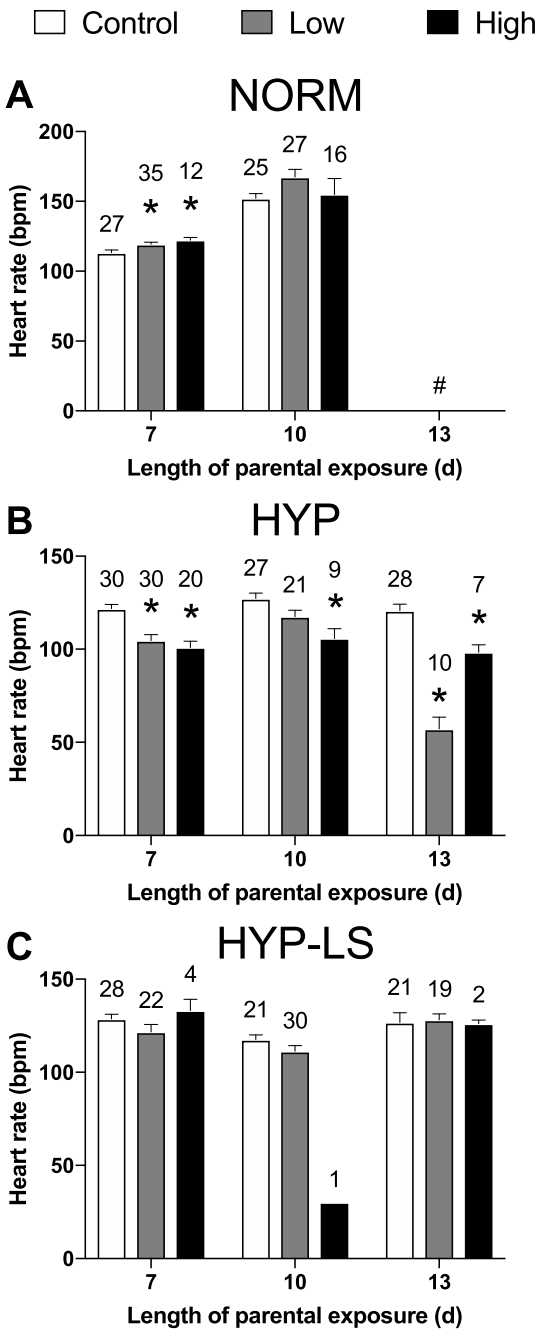

Fig. 2. Heart rate of 2 d post fertilization F_1 embryos following exposure of F_0 sheepshead minnow (SHM) to high-energy water accommodated fraction (HEWAF) under three different environmental scenarios, (A) normoxic (NORM), (B) hypoxic (HYP), and (C) hypoxic with low salinity (HYP-LS). F_0 adult SHM were exposed to 0%, 1.25% (low) or 12.5% (high) HEWAF for 14 d. Heart rate was assessed in embryos collected following 7, 10, and 13 days of parental HEWAF exposure. Asterisks indicate a significant difference from control within environmental scenarios and time of embryos collections. All data are expressed as mean ± standard error and were analyzed using one-way ANOVA with Holm-Sidak test or one-way ANOVA on Ranks with Dunn's test when data violated assumptions ($p < 0.05$). Sample sizes (number of embryos) are indicated by the data labels in the figure. Data for NORM scenario are reprinted from (Jasperse et al., 2019b). "#" = data excluded because of heater malfunction.

than controls ($p = 0.009$, Fig. 2A). F_1-7-HYP-L and F_1-7-HYP-H embryos had heart rates 14% and 17% lower than controls, respectively ($p < 0.001$, Fig. 2B). F_1-10-HYP-H also had 17% lower heart rate compared to controls ($p = 0.004$). Additionally, F_1-13-HYP-L and F_1-13-HYP-H embryos had heart rate 53% and 19% lower than controls, respectively ($p < 0.001$, Fig. 2B). F_1 embryos produced by fish exposed to HEWAF in the HYP-LS scenario had no significant differences in heart rates compared to controls (Fig. 2C). There was only one F_1-10-HYPLS-H larva in which to measure heart rate, and while there was a 74% reduction in heart rate compared to controls, it could not be assessed for statistical significance (n = 1).

3.2. Hatch rate/time to hatch

Embryos produced by fish exposed to HEWAF in the NORM scenario had no significant differences in hatch rates compared to controls (Fig. 3A). There were also no significant effects of parental HEWAF exposure in HYP on F_1 hatch rate on days 7 and 10. However, F_1-13-HYP-L embryos had hatch rate 100% lower than controls ($p = 0.003$, Fig. 3B). Embryos produced by fish exposed to HEWAF in the HYP-LS scenario had no significant differences in hatch rates compared to controls (Fig. 3C).

There were no significant changes from controls in time to hatch in embryos across all exposures in all environmental scenarios. Importantly, hatch rate and time to hatch could not be assessed for tanks in which no embryos were produced, resulting in sample sizes fewer than 6 in some groups. Moreover, time to hatch could not be assessed in tanks for which there was 0% hatch, resulting in different sample size between endpoints.

3.3. Length at hatch (Length0)

F_1-7-NORM-L and F_1-7-NORM-H embryos were approximately 10% shorter at hatch compared to controls ($p = 0.003$, Fig. 4A). There were no significant differences in Length0 of F_1-10-NORM embryos. There were no significant differences in Length0 among F_1-7-HYP and F_1-13-HYP embryos (Fig. 4B). However, F_1-10-HYP-L embryos had 7% shorter Length0 compared to controls ($p = 0.011$). There were no significant differences in Length0 for embryos in the HYP-LS scenario (Fig. 4C).

3.4. Length 10 days post hatch (Length10)

Differing sample size for Length0 and Length10 groups can be attributed to both mortality of larvae before 10 dph, as well as the practical limitations in measuring length0 in embryo cups previously mentioned (see Methods – Developmental Endpoints). F_1-7-NORM-H larvae had 21% longer Length10 compared to controls ($p < 0.001$, Fig. 4D). Additionally, F_1-10-NORM-H larvae had 16% longer Length10 compared to controls, while F_1-10-NORM-L had 17% shorter Length10 compared to controls ($p < 0.001$). F_1-7-HYP-L and F_1-7-HYP-H larvae had 13% and 14% longer Length10 compared to controls, respectively ($p < 0.001$, Fig. 4E). F_1-10-HYP-H larvae also had 45% longer Length10 compared to controls ($p < 0.001$). No F_1-13-HYP-L or F_1-13-HYP-H larvae survived to 10 dph, therefore differences in Length10 could not be assessed in this group (Fig. 4E). F_1-7-HYPLS-H larvae had 39% longer Length10 compared to controls ($p = 0.005$), but there were no significant Length10 changes in F_1-10-HYPLS or F_1-13-HYPLS larvae (Fig. 4F). However, F_1-13-HYPLS-H larvae had Length10 measurements 37% larger than controls, but n = 2 sample size prevented statistical analysis.

3.5. Survival to 10 days post hatch

There were no significant changes in F_1 survival to 10 dph in F_1-7-NORM or F_1-10-NORM larvae (Fig. 5A). There were also no significant changes in F_1 survival among F_1-10-HYP larvae (Fig. 5B). However, the rate of survival to 10 dph in F_1-7-HYP-L larvae was reduced by 29% compared to controls ($p = 0.024$). F_1-13-HYP larvae could not be tested for survival, as both the low and high HEWAF groups had sample

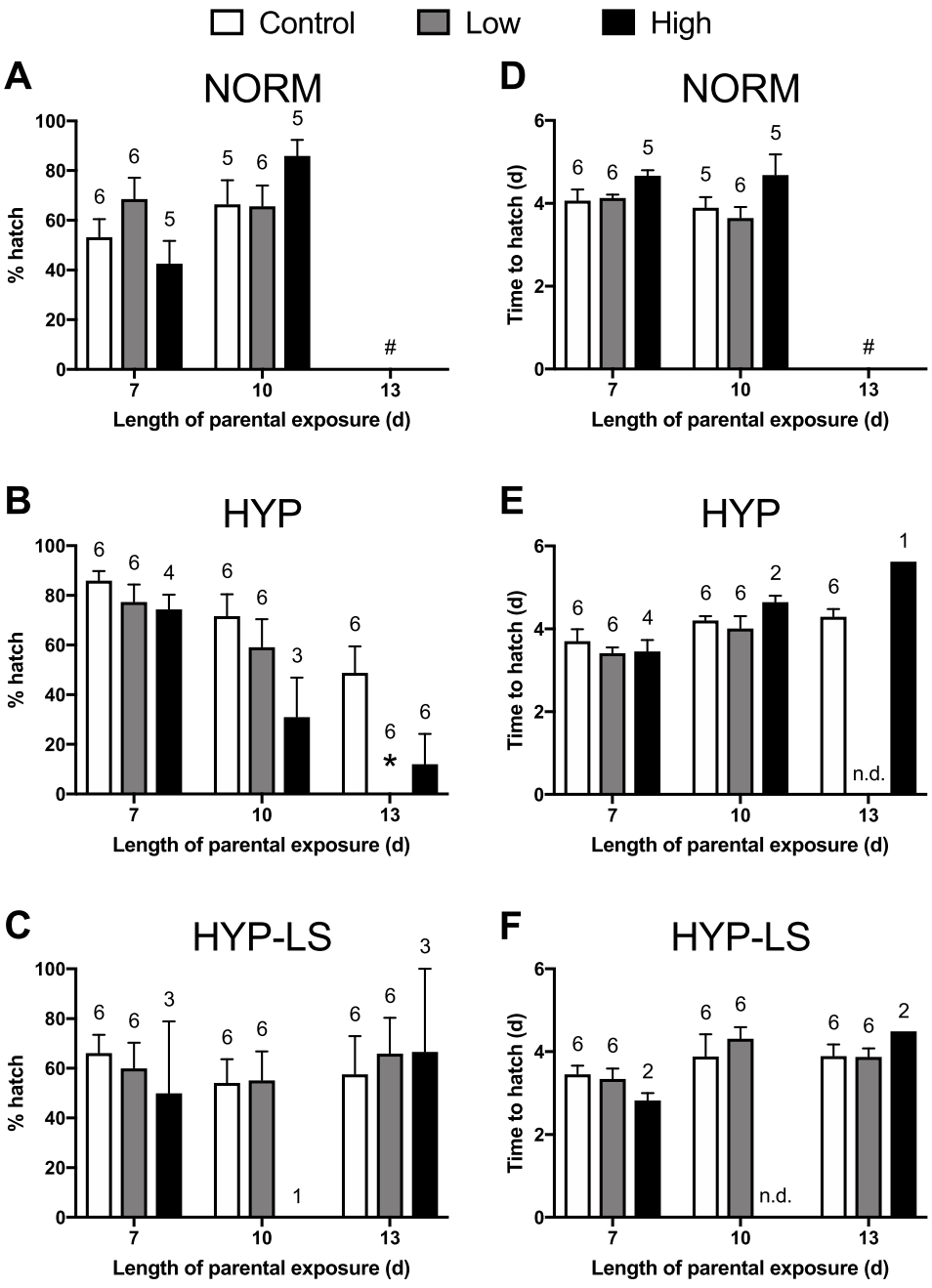

Fig. 3. Hatch rate (A–C) and time to hatch (D–F) of F_1 embryos following exposure of F_0 sheepshead minnow (SHM) to high-energy water accommodated fraction (HEWAF) under three different environmental scenarios, (A, D) normoxic (NORM), (B, E) hypoxic (HYP), and (C, F) hypoxic with low salinity (HYP-LS). F_0 adult SHM were exposed to 0%, 1.25% (low) or 12.5% (high) HEWAF for 14 d. Hatch rate and time to hatch were assessed in embryos collected following 7, 10, and 13 days of parental HEWAF exposure. Asterisks indicate a significant difference from control within environmental scenarios and time of embryos collections. All data are expressed as mean ± standard error and were analyzed using one-way ANOVA with Holm-Sidak test or one-way ANOVA on Ranks with Dunn's test when data violated assumptions ($p < 0.05$). Sample sizes (number of tanks) are indicated by the data labels in the figure. Data for NORM scenario are reprinted from (Jasperse et al., 2019b). "#" = data excluded because of heater malfunction. "n.d." = no data.

size < 3. Of note, the one tank of F_1-13-HYP-H larvae that had any hatching of larvae had a survival rate of 0%, which is substantially lower than the F_1-13-HYP-C larvae survival rate of 83%, although we could not test for statistical significance. There were no significant changes in F_1 survival in the HYP-LS scenario (Fig. 5C).

3.6. Prey capture 10 days post hatch

F_1-7-NORM-H larvae had 80% and 71% lower prey capture compared to controls at 2 min ($p < 0.001$, Fig. 6A) and 5 min ($p < 0.001$, Fig. 6D), respectively. Interestingly, there were no significant differences in prey capture among F_1-10-NORM larvae. There were also no significant differences in prey capture among F_1-7-HYP or F_1-10-HYP larvae (Fig. 6B and E). No F_1-13-HYP-L or F_1-13-HYP-H larvae survived to 10 dph, therefore differences in prey capture could not be assessed. F_1-7-HYPLS-H larvae had 92% lower prey capture compared to controls at 2 min ($p = 0.002$, Fig. 6C), but not 5 min (Fig. 6F). F_1-10-HYPLS-L larvae also had 29% lower prey capture compared to controls at 2 min ($p = 0.031$), but not 5 min. No F_1-10-HYPLS-H survived to 10 dph, therefore differences in prey capture between high HEWAF and controls could not be assessed. There were no significant differences in prey capture among F_1-13-HYPLS larvae.

4. Discussion

The present study demonstrated that adult F_0 SHM exposure to HEWAF resulted in an array of developmental effects in F_1 offspring that were raised in clean seawater through 10 dph. Importantly, due to drastic reduction in egg production by the fish exposed to high HEWAF in the HYP and HYP-LS scenarios (Jasperse et al., 2019a), there were fewer larvae from these groups in which to assess developmental endpoints, which reduced statistical power, when statistical testing was even possible, and may have caused a survivor bias. The results reported in the present study may therefore underestimate the effects of

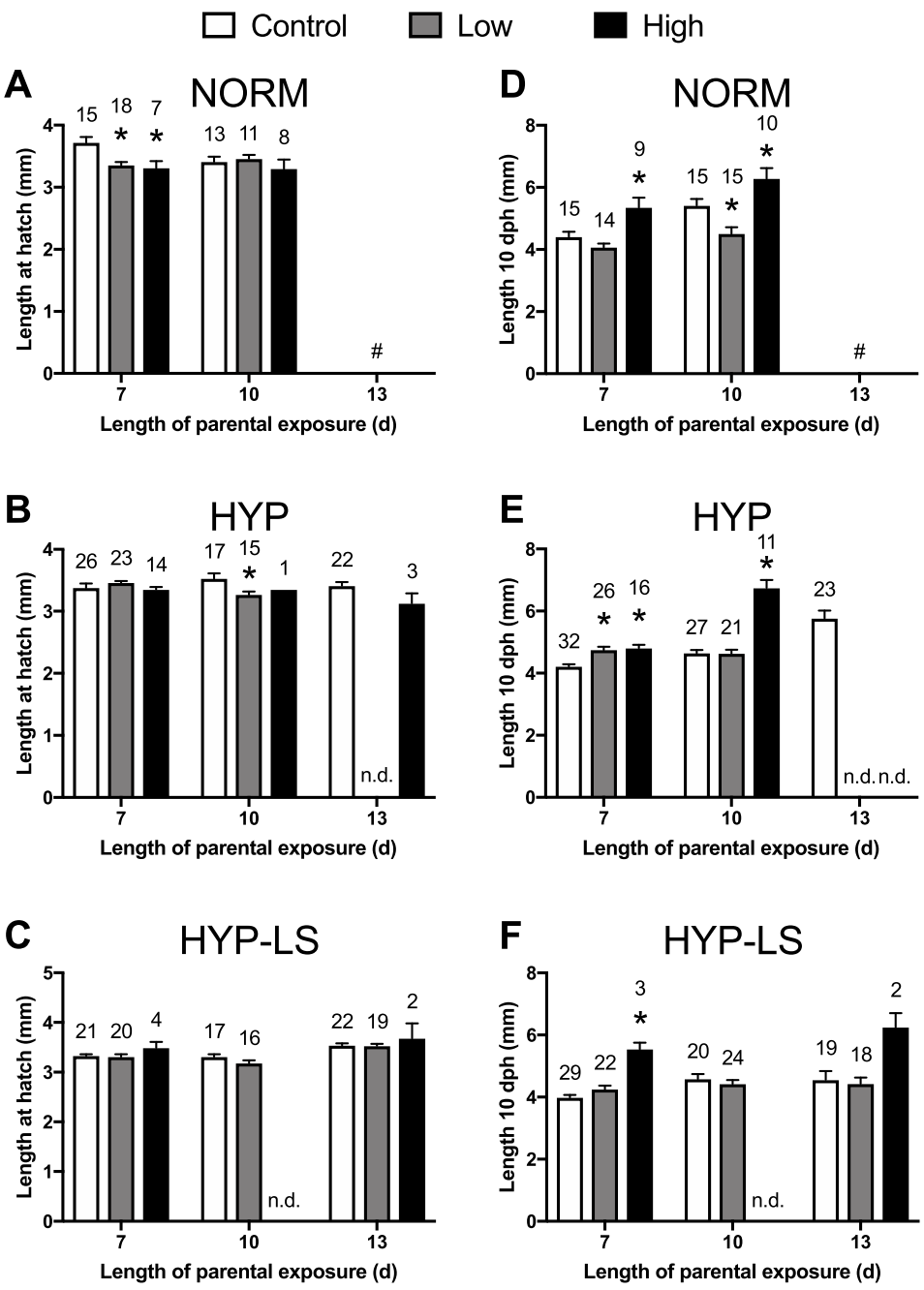

Fig. 4. Length of F_1 embryos at hatch (A–C) and at 10 d post hatch (D–F) following exposure of F_0 sheepshead minnow (SHM) to high-energy water accommodated fraction (HEWAF) under three different environmental scenarios, (A, D) normoxic (NORM), (B, E) hypoxic (HYP), and (C, F) hypoxic with low salinity (HYP-LS). F_0 adult SHM were exposed to 0%, 1.25% (low) or 12.5% (high) HEWAF for 14 d. Lengths were assessed in embryos collected following 7, 10, and 13 days of parental HEWAF exposure. Asterisks indicate a significant difference from control within environmental scenarios and time of embryos collections. All data are expressed as mean ± standard error and were analyzed using one-way ANOVA with Holm-Sidak test, one-way ANOVA on Ranks with Dunn's test when data violated assumptions, or *t*-test when testing for significance between only two groups ($p < 0.05$). Sample sizes (number of embryos) are indicated by the data labels in the figure. Data for NORM scenario are reprinted from (Jasperse et al., 2019b). "#" = data excluded because of heater malfunction. "n.d." = no data.

F_0 high HEWAF exposure in the HYP and HYP-LS scenarios on F_1 development.

Embryos in this study were only directly exposed to HEWAF for a maximum of 24 h (between spawning and daily embryo collection), and therefore may have effects resulting from direct exposure for a short duration (≤24 hpf) and/or from maternal transfer. Both of these processes have previously been documented in fish embryos (McElroy et al., 2006; Monteverdi and DiGiulio, 2000). Maternal transfer of PAHs was previously demonstrated in Atlantic killifish (*Fundulus heteroclitus*), in which benzo[a]pyrene accumulated in maturing oocytes following exposure of gravid females (Monteverdi and DiGiulio, 2000). Some PAHs are suspected to be passed from the mother into the yolk as a method of depuration, and therefore passed onto the offspring (Weis et al., 2003). Maternal transfer of benzo[a]pyrene was speculated to be mediated through association with vitellogenin (Monteverdi and DiGiulio, 2000). PAHs may also pass into the embryo directly from seawater. Japanese medaka (*Oryzias latipes*) embryos were shown to rapidly accumulate and metabolize benzo[a]pyrene from sediment, indicating that some PAHs can pass through the embryo chorion directly (McElroy et al., 2006). It is also possible that epigenetics plays a role in the observed developmental effects, as PAH exposure has previously been demonstrated to change DNA methylation patterns in zebrafish embryos (Corrales et al., 2014).

In the present study, the environmental conditions in which the parental F_0 generation was exposed to HEWAF affected the developmental changes experienced by the F_1 offspring. We previously demonstrated that environmental scenario influenced the bioaccumulation of PAHs in F_0 adults following HEWAF exposure (Jasperse et al., 2019a), and therefore, likely affected the transfer of PAHs or metabolites to the embryos. In general, water concentrations of PAHs were highest in the NORM scenario, while adult fish exposed to HEWAF in the HYP scenario tended to have the highest bioaccumulation of PAHs. The overlap between intracellular oil and hypoxia response signaling pathways (Mandl and Depping, 2014) or suppression of metabolic rate

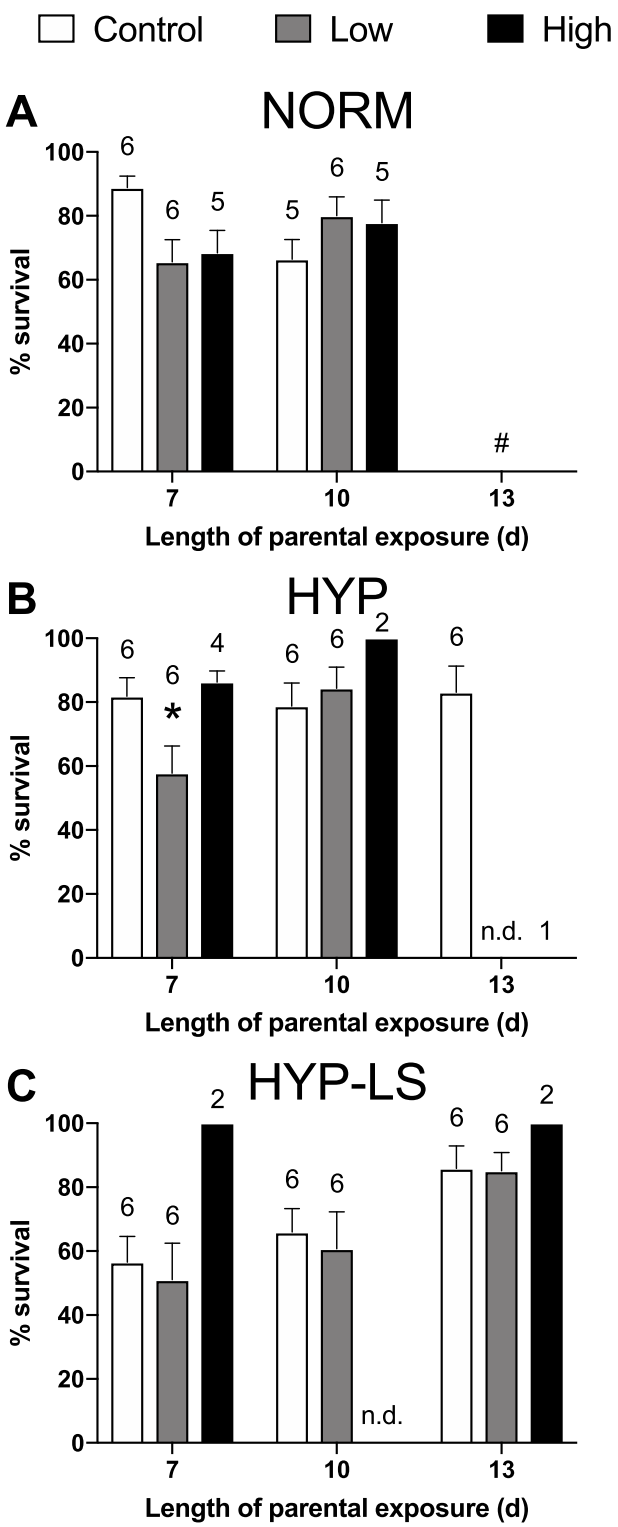

Fig. 5. Survival of F_1 embryos at 10 d post hatch following exposure of F_0 sheepshead minnow (SHM) to high-energy water accommodated fraction (HEWAF) in three different environmental scenarios, (A) normoxic (NORM), (B) hypoxic (HYP), and (C) hypoxic with low salinity (HYP-LS). F_0 adult SHM were exposed to 0%, 1.25% (low) or 12.5% (high) HEWAF for 14 d. Survival was assessed in embryos collected following 7, 10, and 13 days of parental HEWAF exposure. Asterisks indicate a significant difference from control within environmental scenarios and time of embryos collections. All data are expressed as mean ± standard error and were analyzed using one-way ANOVA with Holm-Sidak test or *t*-test when testing for significance between only two groups ($p < 0.05$). Sample sizes (number of tanks) are indicated by the data labels in the figure. Data for NORM scenario are reprinted from (Jasperse et al., 2019b). "#" = data excluded because of heater malfunction. "n.d." = no data.

during hypoxia (Richards, 2009) could result in reduced PAH metabolism under hypoxic conditions, leading to increased partitioning of PAHs to lipid-rich tissues, such as the gonads, in HYP and HYP-LS scenarios. Low salinity in the HYP-LS scenario also may have influenced embryonic exposure to PAHs, as bioavailability and uptake of PAHs tends to increase with decreased salinity (Ramachandran et al., 2006).

The environmental conditions of the F_0 exposure also could have resulted in direct effects on the embryos during the ≤24 hpf that the embryos remained in the F_0 tanks, though it is important to point out that all statistical comparisons were made to the controls within a given environmental scenario, which experienced the same environmental conditions as the HEWAF exposure groups. Hypoxia can be lethal to developing Gulf killifish (*Fundulus grandis*) embryos (Rodgers et al., 2018), though transfer of embryos into aerated water ≤24 hpf in the present study appeared to mitigate this effect, allowing for assessment of developmental endpoints. Interestingly, hypoxia during early development (24 hpf) was shown to alter development of zebrafish embryos by altering patterns of apoptosis (Shang and Wu, 2004).

Heart rate was altered in embryos from two of the three F_0 environmental scenarios (NORM and HYP). Cardiotoxicity is a hallmark developmental effect of PAHs on early life stages of fish, and the heart is often considered the primary target of oil exposure (Incardona et al., 2009). PAHs can induce bradycardia by disrupting excitation-contraction coupling by inhibiting K^+ efflux from the cardiomyocyte and reducing Ca^{2+} influx, resulting in decreased cardiac function (Brette et al., 2014, 2017). Decreased heart rate following embryonic exposure to PAHs was observed in several GOM estuarine species, including SHM, inland silversides (*Menidia beryllina*), yellowfin tuna (*Thunnus albacares*), Atlantic bluefin tuna (*Thunnus thynnus*), mahi-mahi (*Coryphaena hippurus*), and greater amberjack (*Seriola dumerili*) (Bosker et al., 2017; Denslow et al., 2015; Dubansky et al., 2013; Incardona et al., 2014; Perrichon et al., 2018). These studies all involved direct exposure of embryos to PAHs during early development, with tPAH concentrations (~3–150 ng/ml) comparable to levels in F_0 exposure tanks of the present study.

Embryos from parents exposed to HEWAF in the NORM scenario demonstrated increased heart rate compared to controls. While most studies of PAH cardiotoxicity describe a decreased heart rate in response to exposure, increased heart rate has also been reported in marine medaka F_1 embryos collected from F_0 adults exposed to phenanthrene and thereafter raised in clean water (Sun et al., 2015), as well as in zebrafish exposed to phenanthrene during early development (Zhang et al., 2013). The authors speculated that increased heart rate might be a compensatory response to decreased stroke volume to mitigate effects on cardiac output (Zhang et al., 2013). It is therefore plausible that increased heart rate represents an early and less severe manifestation of PAH cardiotoxicity, while decreased heart rate represents a later and more severe manifestation, potentially resulting from a higher dose or longer duration exposure.

Our data suggest an exacerbation of cardiotoxicity in offspring of F_0 exposed to PAHs in an adverse environmental condition (hypoxia). Exacerbation of embryo cardiotoxicity in hypoxia has previously been documented following direct embryo exposure. A study of zebrafish demonstrated that embryos exposed to phenanthrene in hypoxia experienced a more severe reduction in heart rate than embryos exposed in normoxia (Cypher et al., 2017). While embryos from the HYP scenario had drastic bradycardia, there were no significant effects in heart rate of embryos from the HYP-LS scenario. This is likely influenced by a small sample size of embryos of F_1-HYPLS-H in which to measure heart rate, and possible survivor bias. Importantly, a short term (48 h) exposure of mahi-mahi embryos to HEWAF resulted in cardiotoxicity (pericardial edema) and a latent impairment of swimming performance as juveniles, suggesting that early cardiotoxic effects can manifest as physiological impairments at later life stages that may lead to reduced survival in a natural environment (Mager et al., 2014).

Despite no change in time to hatch, hatch rate was decreased in

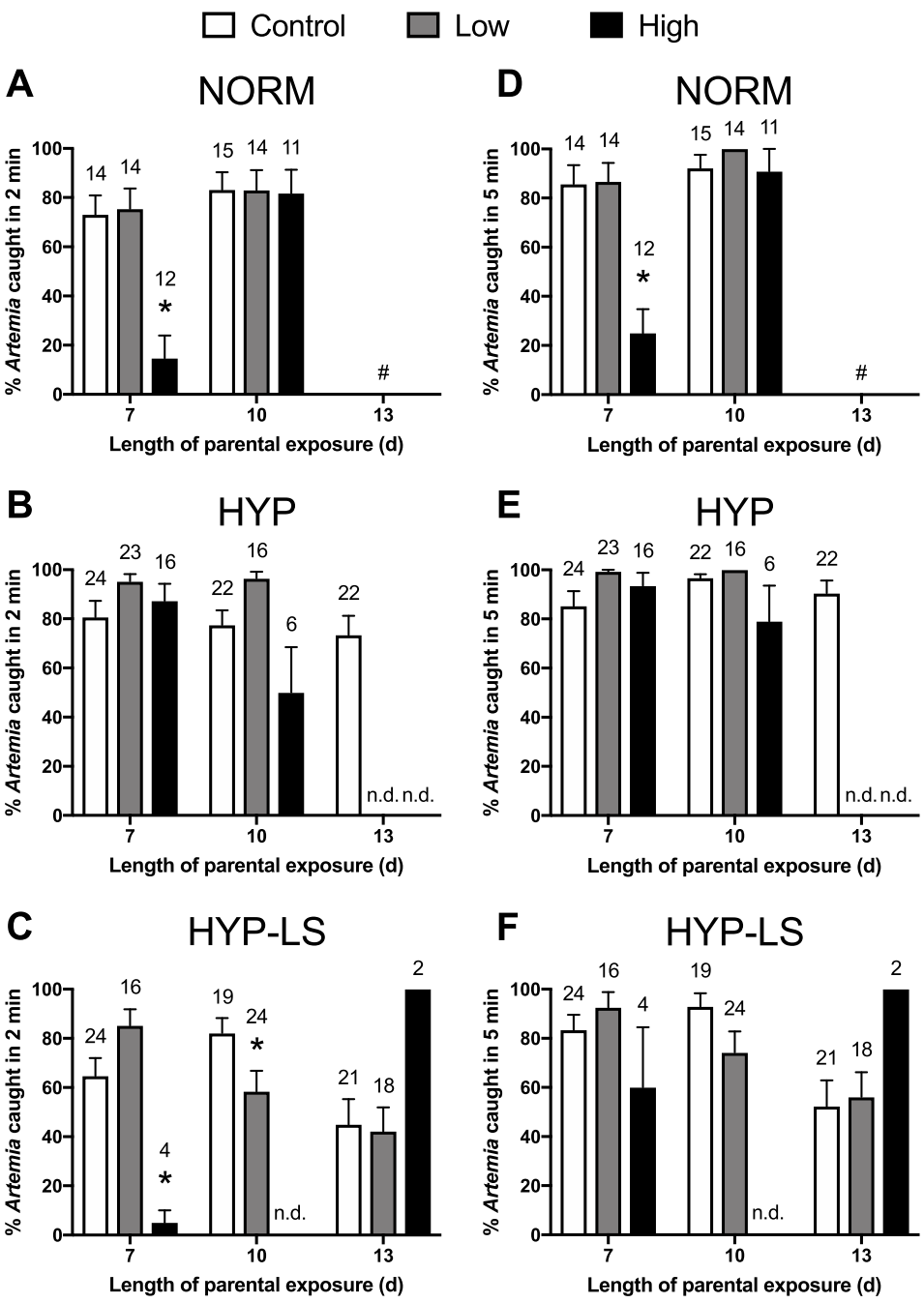

Fig. 6. Prey (*Artemia salina*) capture ability of F_1 embryos at 10 d post hatch following exposure of F_0 sheepshead minnow (SHM) to high-energy water accommodated fraction (HEWAF) under three different environmental scenarios, (A, D) normoxic (NORM), (B, E) hypoxic (HYP), and (C, F) hypoxic with low salinity (HYP-LS). Prey capture was assessed for 2 (A–C) and 5 min (D–F). F_0 adult SHM were exposed to 0%, 1.25% (low) or 12.5% (high) HEWAF for 14 d. Prey capture was assessed in embryos collected following 7, 10, and 13 days of parental HEWAF exposure. Asterisks indicate a significant difference from control within environmental scenarios and time of embryos collections. All data are expressed as mean ± standard error and were analyzed using one-way ANOVA with Holm-Sidak test, one-way ANOVA on Ranks with Dunn's test when data violated assumptions, or *t*-test when testing for significance between only two groups ($p < 0.05$). Sample sizes (number of larvae) are indicated by the data labels in the figure. Data for NORM scenario are reprinted from (Jasperse et al., 2019b). "#" = data excluded because of heater malfunction. "n.d." = no data.

embryos collected from the HEWAF-exposed fish in the HYP scenario, but not the NORM scenario, indicating that hypoxia is essential for the manifestation of reduced hatching success as a result of F_0 HEWAF exposure. While there was no statistically significant reduction in hatch rate in the HYP-LS scenario, all high HEWAF groups had lower sample size, including a sample of only 1 tank for F_1-10-HYPLS-H embryos, which limited the ability to detect statistically significant differences. A previous study continuously exposed Gulf killifish embryos to HEWAF in normoxic conditions and demonstrated that HEWAF exposure causes a dose-dependent decrease in hatch rate, though it was statistically significant only for the highest concentration of HEWAF tested, approximately 4-fold higher than "high HEWAF" in the present study (Rodgers et al., 2018). Additionally, Japanese medaka chronically exposed to PAH-contaminated sediment extract in hypoxic conditions produced F_1 embryos with decreased hatching success, while medaka exposed to the same concentration of sediment extract in normoxic conditions had offspring with no significant changes in hatch rate, demonstrating exacerbation of PAH-induced hatch rate decline (Mu et al., 2017).

Embryos collected from HEWAF-exposed fish in both the NORM and HYP scenarios exhibited decreased length at hatch (Length0), and F_1-10-NORM-L larvae also had reduced length 10 dph (Length10). Decreased length was reported in embryos or larvae exposed to PAHs for several species, including SHM, spotted seatrout (*Cynoscion nebulosus*) and pacific herring (*Clupea pallasi*) (Bosker et al., 2017; Brewton et al., 2013; Carls et al., 1999). Additionally, exposure to hypoxia was shown to significantly decrease body length of zebrafish embryos (Shang and Wu, 2004), and reduced growth was documented following exposure of adult Gulf killifish to hypoxia for 1 month (Landry et al., 2007). A study of Japanese medaka also demonstrated that embryos exposed to hypoxia alone or co-exposed to PAHs and hypoxia both had decreased larval length compared to control embryos (Mu et al., 2017).

Embryo length is closely related to the maternal investment per larva (Johnson et al., 2010), which is likely to be influenced by HEWAF exposure, as female fish would likely need to divert substantial energy to metabolic pathways in the liver. Importantly, size at hatch was shown to be closely related to the probability of survival of the larvae (Garrido et al., 2015). While survival was only significantly lower than control in F_1-7-HYP-L embryos in the present study, it is possible that survival would have been impacted more substantially in a natural environment, where predators and competition for resources would likely influence survival outcome.

Interestingly, while Length0 tended to be decreased or unaffected by F_0 HEWAF exposure, Length10 tended to be increased, especially at high HEWAF concentrations, under all exposure scenarios. While most studies report a decrease in length following PAH exposure, a study of zebrafish observed that embryos from parents with dietary exposure to PAHs had increased larvae size (Perrichon et al., 2015). Importantly, in the present study, Length10 may have been influenced by food abundance or the number of other larvae in the embryo cup. In other words, embryo cups with fewer embryos, due to a combination of decreased egg production and decreased fertilization rates following HEWAF exposure (Jasperse et al., 2019a), had larvae with increased density-dependent access to food, despite all embryo cups having been fed *ad libitum*. These larvae may therefore have had increased Length10 due to indirect external factors (less competition for food and more space to grow), rather than due to a direct developmental change resulting from HEWAF exposure.

Embryos from HEWAF-exposed F_0 generally had survival rates comparable to controls, indicating that neither early life exposure to HEWAF ($\leq$24 hpf) nor maternal effects were directly lethal to the F_1 offspring. Several studies have reported no change in embryo survival following exposure to PAH concentrations that trigger developmental effects (Bosker et al., 2017; Denslow et al., 2015; Dubansky et al., 2013). While the developmental effects shown in the present study are sub-lethal, there is potential for long-term effects of individuals, which could also result in population level effects. It has previously been demonstrated that Atlantic killifish embryos exposed to PAH mixtures experienced long-term behavioral effects that lasted into adulthood (Brown et al., 2016). Early life developmental and behavioral abnormalities can alter organism fitness later in life, and may affect future generations of offspring. We have previously demonstrated that F_1 SHM offspring from HEWAF-exposed F_0 adults generate a F_2 generation with similar deficits in prey capture (Jasperse et al., 2019b). This suggests that parental/early life exposure to PAHs in F_1 SHM can result in long-term effects that manifest in a F_2 generation of offspring that was never directly exposed to oil.

In the present study, prey capture was decreased in embryos collected from HEWAF-exposed fish in both the NORM and HYP-LS scenarios, but not the HYP scenario. The effect of F_0 HEWAF exposure on F_1 prey capture was exacerbated in the HYP-LS scenario, compared to the NORM scenario, as prey capture was reduced to a greater magnitude in embryos collected on day 7 and was impaired following F_0 exposure to a lower concentration of HEWAF (F_1-10-HYPLS-L). Interestingly, while prey capture effects were more severe at 2 min in the HYP-LS scenario, those larvae recovered to normal levels of prey capture by 5 min, while impaired NORM larvae did not. It is possible that F_1-10-HYPLS-H adapted to slower prey capture rates, resulting in reduced prey capture at 2 min, but recovery to normal levels by 5 min. The 2 time points reported for this assay, 2 and 5 min, may simulate environmental situations of limited and abundant prey availability, respectively. Therefore, larvae from F_0 exposed to high HEWAF in the HYP-LS scenario may specifically be sensitive to situations of limited prey availability. It is also possible that parental HEWAF exposure altered the startle-response of larvae, suggesting that a behavioral change may be responsible for impaired prey capture.

Prey capture requires coordination of sensory, behavioral, and locomotor systems (Weis and Weis, 1995), making it a highly sensitive sublethal endpoint to assay. Importantly, normal prey capture is necessary for larval survival in the natural environment, as larvae have a low threshold for starvation (Weis and Weis, 1995). Despite its value as a sensitive endpoint for toxicological assessment, few studies have investigated the effects of PAHs on prey capture of fish. A study of larval Atlantic killifish from contaminated sites found that prey capture was negatively correlated with metals and polychlorinated biphenyls (PCBs), but not PAHs (Weis et al., 2003). In another study, larval stages of dorado (*Salminus brasiliensis*), a carnivorous freshwater fish, exposed to phenanthrene experienced vision impairment and decreased prey capture (Carvalho et al., 2008). Additionally, a study of bluegill (*Lepomis macrochirus*) fingerlings demonstrated that fluorene exposure resulted in decreased prey capture, with reduced proportions of fish striking prey and decreased efficiency of prey capture (proportion of successful prey strikes) (Finger et al., 1985).

The changes in developmental endpoints were generally inconsistent across embryos collection days. This may suggest an impact of duration of F_0 exposure on resultant F_1 developmental effects, or the influence of natural variation of offspring. Interestingly, developmental effects did not worsen as length of parental exposure increased. However, embryos from F_0 exposed to high HEWAF in the HYP and HYP-LS were generally much fewer in number than controls, due to reduced egg production and fertilization in high-HEWAF exposed SHM in HYP and HYP-LS scenarios (Jasperse et al., 2019a). This was particularly problematic in the day 13 embryos collection, which resulted in very few larvae in which to measure developmental effects. Moreover, effects of HEWAF exposure on early developmental endpoints, such as hatch rate, reduced the sample size for subsequent endpoints at later stages of development, such as prey capture. This limited the ability to test for statistical significance (when $n < 3$) or otherwise reduced statistical power, and made it more difficult to determine exacerbation of effects. It is therefore possible that the developmental effects reported in the present study represent an underestimate of the actual risk of HEWAF exposure in adverse environmental scenarios on SHM offspring development.

In summary, the F_1 developmental effects varied depending on the environmental scenario of the F_0 HEWAF exposure, and the effects were exacerbated in scenarios of adverse environmental conditions for some developmental endpoints (heart rate, hatch rate, prey capture), but not for others (Length0, Length10). Nevertheless, the present study demonstrates that HEWAF causes developmental changes in F_1 embryos, which could result in impaired organism fitness in later life stages. The exacerbation of several developmental effects in adverse environmental scenarios suggests that SHM exposed to oil in estuaries experiencing hypoxia or low salinity may produce offspring with worsened outcomes. Moreover, the developmental effects observed in the present study in response to parental HEWAF exposure are compounded by the reproductive effects of HEWAF (reduced egg production and fertilization) previously described (Jasperse et al., 2019a). In other words, adult SHM exposure to HEWAF not only reduces egg production and fertilization, but the embryos produced may further experience cardiac alterations, reduced hatching success, smaller size at hatch, reduced prey capture, and reduced survival, which altogether could lead to serious long-term population level impacts for this species.

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R2.x213.000:0003 (DOI:10.7266/N74M92WR).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2019.104762.

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