L. Jasperse et al.

Transgenerational effects of PAHs on sheepshead minnows

Transgenerational Effects of Polycyclic Aromatic Hydrocarbon Exposure on Sheepshead Minnows (*Cyprinodon variegatus*)[1]

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Abstract

The Deepwater Horizon oil spill resulted in the release of over 640 million L of crude oil into the Gulf of Mexico, affecting over 2000 km of shoreline, including estuaries that serve as important habitats and nurseries for aquatic species. *Cyprinodon variegatus* (sheepshead minnow) are small-bodied fish that inhabit northern Gulf of Mexico estuaries, are easily adaptable to laboratory conditions, and are commonly used in toxicological assessment studies. The purpose of the present study was to determine the somatic, reproductive, and developmental effects of an environmentally relevant polycyclic aromatic hydrocarbon (PAH) mixture, the oil high-energy water accommodated fraction (HEWAF), on experimentally exposed sheepshead minnow (F_0) as well as 2 generations of offspring (F_1 and F_2) without additional exposure. The F_0 generation exposed to HEWAF had increased liver somatic indices, altered egg production, and decreased fertilization. Several developmental endpoints in the F_1 were altered by F_0 HEWAF exposure. Adult F_1 demonstrated decreased weight and length. Both the F_1 and F_2 generations derived from high HEWAF-exposed F_0 had deficits in prey capture compared to control F_1 and F_2, respectively. Correlations between endpoints and tissue PAHs provide evidence that the physiological effects observed were associated with hydrocarbon exposure. These data demonstrate that PAHs were capable of causing physiological changes in exposed adult sheepshead minnow and transgenerational effects in unexposed offspring, both of which could have population-level consequences.

This article contains online-only Supplemental Data.

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INTRODUCTION

The explosion of the Deepwater Horizon oil exploration platform on 20 April 2010 initiated the release of approximately 640 million L of crude oil into the Gulf of Mexico, creating oil slicks that covered more than 100 000 km^2 of the ocean surface (Beyer et al. 2016). More than 2000 km of shoreline were affected, including estuaries that serve as important habitats and nurseries for aquatic species (Beyer et al. 2016). By the time the wellhead was officially sealed on 19 September 2010, an estimated 1.7×10^{11} g of C_1–C_5 hydrocarbons were released into Gulf of Mexico waters (Reddy et al. 2012), reaching shorelines along the coasts of Texas, Louisiana, Mississippi, Alabama, and Florida (Nixon et al. 2016).

Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants of particular concern in oil spills because of their toxicity and carcinogenicity in humans and wildlife (Allan et al. 2012). They enter marine environments through oil spills and combustion of fossil fuels (Walker et al. 2012). The structure and molecular size of PAHs determine both their toxicity and bioavailability in the water column. Smaller and lighter PAHs are more water-soluble and therefore more readily taken up by marine organisms through the gills or the digestive tract (Hylland 2006; Walker et al. 2012). Heavier PAHs are less soluble and tend to partition into lipid-rich tissues (Shukla et al. 2007). Once inside the host, PAHs can be metabolized by cyclooxygenases, such as cytochrome p450 1a1, into polar metabolites and then into conjugates, which are more readily excreted (Ma and Lu 2007; Walker et al. 2012).

Although many studies have established the toxicity of hydrocarbons (individual or mixtures) on fish reproductive and developmental functions (Villeneuve et al. 2001; Incardona et al. 2004; Reynaud and Deschaux 2006; Dubansky et al. 2013; Booc et al. 2014; Brown-Peterson et al. 2015; Beyer et al. 2016; Hedgpeth and Griffitt 2016; Raimondo et al. 2016), few have investigated multi- or transgenerational effects. Furthermore, most generational studies use a chronic exposure across all generations tested, which may not be an environmentally relevant parallel to exposure during an oil spill when only the parental generation (F_0) is exposed. To date, there have been only 4 studies that have investigated the effects on unexposed generations of offspring following F_0 exposure (transgenerational effects; White et al. 1999; Corrales et al. 2014; Perrichon et al. 2015; Raimondo et al. 2016). Although these studies have demonstrated transgenerational effects following hydrocarbon exposure, they either focus on one specific hydrocarbon, limiting the environmental relevance of the exposure, or restrict their transgenerational observations to the early life stages of the F_1 generation. There is a need for more extensive research on the transgenerational effects of environmentally relevant hydrocarbon mixtures, specifically with more extensive evaluation of reproductive endpoints in the F_1 generation, as well as monitoring the F_2 generation.

In the present study, F_0 adult sheepshead minnow were exposed to a crude oil high-energy water accommodated fraction (HEWAF), an environmentally relevant PAH mixture, and analyzed for somatic and reproductive changes. The F_1 generation was examined for developmental, somatic, and reproductive effects; and the unexposed F_2 generation was evaluated for developmental effects. The present study tested the following hypotheses: 1) oil exposure will impact somatic, reproductive, and developmental endpoints in adult F_0 sheepshead minnow and 2) oil exposure will result in transgenerational effects on F_1 and F_2 generations.

These hypotheses were tested using the following objectives: 1) to determine the effect of oil exposure on somatic, reproductive, and developmental endpoints; 2) to determine the correlation between body burden of PAHs and somatic and reproductive endpoints; and 3) to determine changes in somatic and reproductive endpoints in the adult F_1 generation and changes in developmental endpoints in the unexposed embryonic F_2 generation.

METHODS

Test species

Sheepshead minnow, *Cyprinodon variegatus*, are an abundant small-bodied fish (<8 cm long) found in shallow waters along the Atlantic coast of the United States, from Cape Cod to Mexico (Bigelow and Schroeder 1953), including estuaries along the northern Gulf of Mexico coastline. Furthermore, sheepshead minnow produce transparent demersal eggs, allowing for easy assessment of embryonic developmental endpoints. Their hardiness, environmental relevance and abundance, adaptability to laboratory conditions, and short developmental period made them an ideal species for the present study.

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

Fish holding

Fish were housed in the University of Connecticut's Aquatic Facility in 35-L glass aquaria and/or a 1890-L Mini Fish Farm (Pentair Aquatic Ecosystems). Routine water quality tests were performed, including daily testing for dissolved oxygen, temperature, and salinity and weekly testing for pH, ammonia, nitrite, and nitrate. All measurements of pH, ammonia, nitrite,

and nitrate were within normal ranges. During housing, adult fish were fed standard commercial flaked food once a day (Zeigler Aquatox Fish Diet; Zeigler Bros.). During reproductive experiments, adult fish were fed flaked food twice a day and freshly hatched brine shrimp (*Artemia salina*) nauplii once a day.

HEWAF preparation

The HEWAF was prepared according to protocols previously described (Incardona et al. 2013). The HEWAF has been demonstrated to produce a chemical composition more similar to whole oil than traditional water accommodated fraction methods can generate (Sandoval et al. 2017). Furthermore, dilutions of HEWAF preparations have been shown to preserve chemical composition across a wide range of dilutions (Forth et al. 2017). Briefly, surrogate oil (BP-AECOM) was mixed with 3 L artificial seawater (Instant Ocean®) in a Waring CB15 high-speed commercial blender at 1 g oil/L seawater for 1 min on low speed. The HEWAF mixtures were prepared in 7 batches (21-L HEWAF), poured into a 23-L glass carboy, and allowed to settle for 1 h. A peristaltic pump was used to collect HEWAF from the carboy, avoiding the oil–water interface. The HEWAF was mixed with artificial seawater in header tanks to produce dilutions (v/v) of 0% (no HEWAF, control), 1.25% (low HEWAF), and 12.5% (high HEWAF), which flowed into individual exposure tanks by passive flow. The flow-through system required approximately 80 L of HEWAF daily; therefore, several 21-L carboys of HEWAF were prepared each day of the reproductive test.

Chemical analyses

For a subset of HEWAF preparations ($n = 5$), a 500-mL sample was kept for chemical analysis by gas chromatography tandem mass spectroscopy (GC-MS/MS) at the University of Connecticut's Center for Environmental Science and Engineering to determine the PAH

composition using methods previously described (Rodgers et al. 2018). Briefly, HEWAF samples were passed across a methanol-conditioned Waters HLB solid-phase extraction cartridge, eluted with acetonitrile, then analyzed using an Agilent 6890 GC with a Restek Rxi-5Sil MS column using splitless injection coupled to a Waters Quattro Micro tandem MS. An internal standard was used to quantify all peaks, and efficiency of extraction was assessed using surrogate standards. Standard quality assurance procedures were utilized, including analysis of duplicate samples, method blanks, matrix spike duplicates, and laboratory control samples. Reporting limits are shown in Supplemental Data, Table S1.

To evaluate the hydrocarbon exposure in the experimental system, a 9-mL sample of water was collected daily from each tank and added to a 20-mL glass scintillation vial containing 9 mL ethanol, which was then stored at 4 °C. Water samples were sonicated for 3 min to reduce PAH adhesion to the glass vial and then analyzed on a fluorescence spectrophotometer using an excitation wavelength of 270 nm and an emission range of 280 to 500 nm to detect total petroleum hydrocarbons (TPHs), including 2- to 4-ring aromatic hydrocarbons (Kim et al. 2010). This fluorescence method of measuring TPHs is a fast and cost-effective alternative to GC/MS that has been routinely used to monitor and characterize oil exposure (Kim et al. 2010).

Experimental conditions—Reproductive test

Adult sheepshead minnow (>120 d posthatch [dph]) were randomly allocated to 20-L glass aquaria with 3 females and 2 males per tank (Cripe et al. 2009), as assessed by sexually dimorphic coloration. Tanks were set up on a flow-through system, similar to systems previously described (Manning et al. 1999). The flow-through system facilitates 2 turnovers of water per day, which allows maintenance of water quality as well as consistent exposure levels of HEWAF.

Prior to the start of the experiment, the fish were acclimated to laboratory conditions for 10 d. Fish were maintained on a 16:8-h light: dark cycle (Manning et al. 1999), and water was kept at 29.8 ± 0.2 °C, dissolved oxygen at 5.57 ± 0.87 mg/L, and salinity at 15 ± 1 ppt. These conditions were chosen based on published sheepshead minnow exposures at 15 ppt salinity (Brown-Peterson et al. 2013; Hedgpeth and Griffitt 2016) and normoxic dissolved oxygen conditions. Temperature was controlled by placing the tanks in a heated water bath, which was monitored daily. Artificial seawater was prepared using Instant Ocean sea salt. Salinity was determined using a Sybon Opticon Series FG100sa refractometer.

After acclimation to the environmental conditions, a 10-d pre-exposure was performed to establish baseline egg production and determine exposure groups (Bosker et al. 2009). During this pre-exposure period, one breeding net made of a PVC ring with fine nylon mesh (335 μm) was placed into each tank to provide a spawning substrate for female fish. The breeding nets were removed daily, allowing for enumeration of eggs in each tank, then rinsed to remove eggs, and returned to the tank. Tanks with actively spawning fish were selected for the exposure and distributed among treatment groups (6 tanks per treatment). During this pre-exposure period, eggs were not assessed for fertilization or hatching success.

Reproductive and developmental endpoints

Egg production data were expressed per female to account for discrepancies in the ratio of 3 females to 2 males, from either mortality of a fish during exposure or misidentification of sex based on dimorphic coloration. More specifically, adult male fish lacking the traditional blue coloration and caudal fin black stripe of mature male sheepshead minnow were on occasion misidentified as females during tank assignment. Sex was confirmed during necropsy when

reproductive organs could be dissected and visualized, and egg production values from each tank were then divided by the number of females in each tank.

Six tanks were assigned to each of 3 exposure groups based on average egg production over the 10 d (control 25.3 ± 3.4, low HEWAF 26.1 ± 2.9, high HEWAF 27.0 ± 3.2). Tank assignments were performed following guidelines set forth in Bosker et al. (2009) to ensure that any changes in egg production between groups during the exposure phase were attributable to the HEWAF, not to natural variation in egg production between fish.

Eggs were collected daily from breeding nets throughout the 14-d exposure to determine cumulative egg production during exposure. On days 7 and 10 of the exposure, eggs were kept in embryo cups (a cylinder of nylon mesh adhered to a Petri dish) in tanks with clean, aerated artificial seawater for determination of fertilization rate, as well as developmental endpoints. A maximum of 50 eggs were placed per embryo cup, with one embryo cup per tank. Fertilization rate was determined 2 d after egg collection by visualizing developing embryos using an inverted microscope (Axiovert 200M; Zeiss).

Eggs used to determine F_0 fertilization success were maintained for 10 dph in tanks of clean, aerated seawater to assess developmental endpoints, including heart rate, percentage of hatch, time-to-hatch, larval length at hatch, larval length at 10 dph (LL10), prey capture at 10 dph, and survival. Heart rates were determined 2 d after egg collection using an inverted microscope ($\times$25 magnification) and counting heart beats over a 30-s period. Eggs were then returned to embryo cups and monitored daily for hatching to determine percentage of hatch and time-to-hatch. Once hatched, larvae were collected and photographed on a microscope to determine standard length using AxioVision 4.8.1 software (Zeiss) and then returned to the embryo cups. At 10 dph, larvae were counted to determine survival and photographed on the

microscope to determine length. Prey capture ability was also assessed in the larvae at 10 dph, in a manner similar to methods previously described (Weis et al. 2003). Briefly, larvae were placed individually into a well of a 48-well plate containing 5 or 6 brine shrimp, with a minimum of 10 replicates per exposure. The number of remaining brine shrimp was monitored at 2 and 5 min to determine ability to capture prey.

Somatic endpoints

Following the 14-d exposure, F_0 fish were netted and anesthetized using buffered tricaine methanesulfonate (MilliporeSigma) at a concentration of 0.1 g/L. Standard length and wet weight were determined, and subsequently fish were euthanized by spinal severance (American Veterinary Medical Association 2013). After euthanasia, fish were dissected to remove the liver and gonads, which were weighed for the calculation of liver somatic index (LSI) and gonad somatic index (GSI) for each individual fish. Somatic index was calculated as (organ wt/body wt) × 100.**<ZAQ;1>**

PAH body burden

Fish carcass samples (without livers and gonads) were analyzed for 16 parent PAHs and 8 alkyl PAHs. The PAHs were extracted using the quick, easy, cheap, effective, rugged, and safe (QuEChERs) method (Johnson 2012) in water, followed by addition of acetonitrile, cleanup with magnesium sulfate and sodium acetate, and centrifugation. Extracts were then analyzed by ultra-performance liquid chromatography/tandem mass spectrometry photodiode array detection, as described (Paruk et al. 2013; Yeudakimau et al. 2013; Seegar et al. 2015). All quality control data were within acceptable limits. Reporting limits of parent PAHs are listed in Supplemental Data, Table S1. Bioconcentration factors (BCFs) were calculated as described (Jonsson et al.

2004) using the equation $BCF = C_F/C_W$, where C_F is the PAH concentration measured in fish tissue (without liver and gonads) and C_W is the concentration of PAHs in the seawater.

F_1 generation—somatic and reproductive endpoints

Eggs collected during the F_0 generation exposure were used to measure reproductive and developmental endpoints and maintained in clean, artificial seawater through development to the adult stage (125 dph). The F_1 generation was only directly exposed to HEWAF during the period between when eggs were spawned and when eggs were removed during daily egg collection (<24 h). Somatic and reproductive endpoints were assessed using the same reproductive test as described **<ZAQ;2>**above but without any additional exposure to HEWAF. In addition, the pre-exposure phase used to determine baseline egg production was extended from 10 to 14 d because many of the tanks did not produce any eggs for the first several days. The control F_1 group had 7 replicate tanks for the reproductive test, whereas the low and high HEWAF F_1 groups each had 6 replicate tanks.

F_2 generation

Eggs (F_2) collected from each replicate tank during the F_1 generation reproductive test on days 7, 10, and 13 were maintained in clean artificial seawater (maximum of 50 eggs per tank) to determine developmental endpoints, as described **<ZAQ;3>**above. Because the F_1 reproductive test did not include oil exposure, developmental endpoints were not expected to vary between the 3 egg collections. Therefore, data for F_2 developmental endpoints were combined across egg collections to achieve a higher sample size for statistical analyses.

Statistical analyses

One-way analyses of variance (ANOVAs) with Dunnett's test were used to compare differences between exposed and nonexposed fish in somatic and reproductive endpoints, as well

as developmental endpoints in the F_2 generation. Normality was tested using the Kolmogorov-Smirnov test, and equal variance was assessed with the Levene median test. A one-way ANOVA on ranks was used when data violated normality assumptions. A 2-way ANOVA with the Holm-Sidak test was used to compare differences in fertilization success and all developmental endpoints for the F_1 generation.

Body burdens of parent and alkyl PAHs were used for correlation analyses with all experimental endpoints. Body burden data were pooled separately for males and females of each tank. Control fish were not included in the correlation analyses. Correlation analyses of PAH body burdens and experimental endpoints were performed using Pearson's product moment correlation. All analyses were performed using SigmaStat 3.5 software (Systat Software), using an alpha level of 0.05 for statistical significance. All data are presented as mean ± standard error of the mean, unless indicated otherwise.

RESULTS

Chemical analyses

Total PAHs (tPAHs) of the 5 HEWAF samples averaged 136.9 ± 30 ng/mL (mean ± standard deviation, Table 1). Based on this measured mean tPAH concentration, the 1.25% HEWAF dilution ("low HEWAF") was determined to be 1.7 ng/mL tPAH and the 12.5% HEWAF dilution ("high HEWAF"), 17 ng/mL tPAH. Daily quantification of TPH in individual tanks of fish measured using fluorescence indicated that exposure remained steady throughout the 14-d exposure and consistent between tanks of the same dilution (Supplemental Data, Table S2). Also, fluorescence values indicated that the high HEWAF exposure was approximately 11 times higher in TPH concentration compared to the low HEWAF exposure (low HEWAF, 75 au; high HEWAF, 807 au).

Body burdens of parent PAHs and alkyl PAHs in F_0 sheepshead minnow following the 14-d HEWAF exposure are summarized in Table 1. Fish from control tanks were pooled by tank, and all PAHs analyzed were below the limit of detection. Body burdens of PAHs were relatively similar in composition to the stock HEWAF; the most abundant PAHs were naphthalene, fluorene, anthracene, and several methylated naphthalene alkyl PAHs (Table 1).

Bioconcentration factors (based on whole fish without livers and gonads) are reported in Supplemental Data, Table S3. In both low and high HEWAF exposures, BCFs varied widely among hydrocarbons, ranging from 0 to over 18 000, though BCFs tended to be less variable for alkyl PAHs than they were for the parent PAHs. The BCFs were larger in low HEWAF F_0 than high HEWAF F_0 for the parent PAHs naphthalene and fluorene, as well as 3 of 8 alkyl PAHs. Alternatively, BCFs were larger in high HEWAF F_0 than low HEWAF F_0 for the parent PAHs acenaphthene, phenanthrene, anthracene, chrysene, benzo[*a*]anthracene, as well as 4 of 8 alkyl PAHs.

F_0 generation—somatic endpoints

During the 14-d HEWAF exposure, minimal mortality of adult sheepshead minnow was observed. Survival rates were 100, 93, and 97% for control, low, and high HEWAF fish, respectively. Following a 14-d HEWAF exposure, sheepshead minnow had no significant differences in length, body weight, gonad weight, or GSI (Table 2). However, female fish exposed to high HEWAF for 14 d had significantly higher liver weight than unexposed female fish, and both males and females exposed to high HEWAF had significantly higher LSI than unexposed fish. Liver somatic indices were positively correlated with tissue tPAHs for 4 of the 7 parent PAHs detected in tissues, as well as for 6 of the 8 alkyl PAHs (Table 3).

F_0 generation—reproductive endpoints

There were no significant differences in average daily egg production between exposure groups in the pre-exposure period (Figure 1A). However, during the exposure period daily egg production was significantly lower in high HEWAF exposure compared to low HEWAF exposure (Figure 1A). Moreover, high HEWAF exposure significantly reduced the average egg production per day compared to the same tanks during the pre-exposure period (Figure 1A). After a 14-d exposure period, the cumulative egg production per female averaged 350 ± 31 (mean $\pm$ standard error) for controls, 444 ± 36 for low HEWAF, and 234 ± 36 for high HEWAF (Figure 1B). Low HEWAF exposure resulted in a 27% increase in cumulative egg production compared to control fish, but this was not statistically significant (Figure 1B). High HEWAF exposure resulted in a 33% decrease in cumulative egg production, but this was significantly different only from low HEWAF, not control (Figure 1B).

Eggs collected on days 7 and 10 of exposure were kept for evaluation of egg fertilization rate, a measure of male reproductive capacity. Overall, fertilization success was significantly lower in high HEWAF eggs compared to low HEWAF eggs, although neither was significantly different from control (Figure 1C). In addition, among eggs collected on day 7 of exposure only, there was a significant decrease in fertilization rate in eggs from high HEWAF fish compared to low HEWAF. Fertilization rate on day 7 of exposure was negatively correlated with tissue tPAHs, including 4 of the 7 parent PAHs detected in tissues as well as all 8 alkyl PAHs (Table 3).

F_1 generation—developmental endpoints

There were no statistically significant changes in larvae survival to 10 dph, which ranged between 65 and 89% (data not shown). Among embryos collected on day 10, the heart rate of embryos exposed to low HEWAF was significantly higher (by 10%) than it was for control

embryos (Figure 2A). In addition, heart rate was 34% higher in embryos collected on day 10 compared to embryos collected on day 7 in all 3 exposure groups. The HEWAF treatment and time of egg collection had a significant interaction on embryo hatch rate (Figure 2B). Within the high HEWAF exposure, embryos on day 7 of exposure had a 48% lower hatch rate than embryos collected on day 10. Time-to-hatch was not significantly affected by HEWAF exposure or time of egg collection (Figure 2C).

Among eggs collected on day 7, embryos from low and high HEWAF exposures were significantly shorter at hatch (larval length at hatch) than control embryos, with reductions of 10 and 11%, respectively (Figure 2D). Overall, high HEWAF exposure also reduced larval length at hatch by 7% compared to controls. In addition, control embryos from eggs collected on day 10 were significantly shorter at hatch (8% reduction) than control embryos from eggs collected on day 7.

Low HEWAF exposure significantly reduced LL10 by 13%, whereas high HEWAF exposure resulted in an 18% increase in larval length (Figure 2E). Within eggs collected on day 7, high HEWAF exposure significantly increased LL10 by 21% compared to control and by 31% compared to low HEWAF exposures. Within eggs collected on day 10, low HEWAF significantly decreased LL10 by 17%, whereas high HEWAF significantly increased length by 16%. In addition, there was an overall effect of time of egg collection because embryos from eggs collected on day 7 of exposure were significantly shorter at 10 dph than those from eggs collected on day 10, with a reduction in length of 15%. This was also statistically significant within control and high HEWAF exposures individually.

The HEWAF exposure and day of egg collection had a significant interaction on prey capture ability of F_1 larvae. Within eggs collected on day 7 of exposure, prey capture ability of

larvae from high HEWAF tanks was 80% lower than that of larvae from control tanks during the first 2 min of the assay and 71% lower than that of control larvae after 5 min, both of which were statistically significant reductions in prey capture (Figure 3B).

F_1 generation—somatic endpoints

There were no statistically significant differences in survival rates of F_1 to 10 dph from the 3 different F_0 groups (data not shown). Of the F_1 larvae that survived, 77, 84, and 95% survived to the adult stage (125 dph) in the control, low HEWAF, and high HEWAF groups, respectively.

There was minimal mortality during the F_1 reproductive test. Only one male fish died on day 13 in the high HEWAF group. Analyses of somatic endpoints at the end of the 14-d reproductive test indicated that female F_1 from the low HEWAF group were significantly smaller compared to F_1 controls (Table 4). There were no statistically significant changes in liver or gonad weight or somatic indices.

F_1 generation—reproductive endpoints

During the 14-d reproductive test, the cumulative egg production per female averaged 50 ± 17 for F_1 fish from F_0 controls, 36 ± 21 for F_1 fish from F_0 low HEWAF, and 24 ± 6 for F_1 fish from F_0 high HEWAF (Figure 4). Although there were 29 and 51% decreases in F_1 egg production in the low and high HEWAF groups, respectively, this effect was not statistically significant ($p = 0.521$), though low power of the ANOVA limits the interpretation of these data.

F_2 generation—developmental endpoints

In the F_2 generation, there were no statistically significant changes in heart rate, hatch rate, time to hatch, length at hatch, length 10 dph, or survival to 10 dph (data not shown). However, prey capture ability of larvae from the high HEWAF group was significantly reduced

compared to larvae from the control and low HEWAF groups after 2 min (Figure 5), but there were no significant differences among groups at 5 min.

DISCUSSION

The present transgenerational study demonstrated that exposure of sheepshead minnow to HEWAF resulted in somatic and reproductive effects in a PAH-exposed F_0 generation, developmental changes in the F_1 generation, and minimal developmental effects in the unexposed F_2 generation. The PAH exposure levels used in the present study were 1.7 and 17 µg/L for low and high HEWAF, respectively, which fall within the range of concentrations measured in the Gulf of Mexico during and after the Deepwater Horizon oil spill, as reported in the largest publicly available database of water chemistry data from the Gulf of Mexico (BP Gulf Science Data 2016). The HEWAF exposures performed in the present study therefore represent environmentally relevant exposures of Gulf of Mexico sheepshead minnow, in terms of both composition and concentration.

Body burdens of PAHs were analyzed only at the end of the 14-d exposure and, therefore, only provide a snapshot of exposure levels inside the fish. It is likely that actual PAH burdens fluctuated throughout the exposure because PAHs were absorbed, metabolized, and excreted (Heath 2018). Importantly, liver and gonad tissues were removed for further molecular analyses and, therefore, could not be included in hydrocarbon body burden analyses. The reported concentrations in the present study may therefore underestimate the actual body burden of exposed fish. Moreover, removal of gonads before PAH analyses limited the ability to make appropriate comparisons of body burden between males and females. Adult sheepshead minnow tended to have higher concentrations of alkyl PAHs than parent PAHs in their tissues. Alkyl PAHs are generally more lipophilic than parent PAHs and, therefore, experience increased

absorption and bioaccumulation (Irwin et al. 1997). In addition, most alkyl PAHs are more toxic than the parent compound (Irwin et al. 1997). Body burdens of tPAHs were approximately 10 times higher in fish exposed to high HEWAF (12.5% stock) than fish exposed to low HEWAF (1.25% stock), indicating that overall body burdens of tPAHs were proportional to water concentrations of tPAHs, at least for the 2 concentrations of HEWAF tested.

Our data bolster the growing body of evidence that hydrocarbon exposure can alter egg production in female fish (Brown-Peterson et al. 2013; Perrichon et al. 2015; Raimondo et al. 2016) and decrease reproductive fitness in male fish by reducing fertilization rate (Booc et al. 2014). Hydrocarbons have also been shown to reduce sperm quantity (Sundt and Bjorkblom 2011) and quality (Nagler and Cyr 1997) and delay spermiation (Khan 2013), though those endpoints were not assessed in the present study. Correlation analyses revealed that tissue concentrations of parent and alkyl PAHs were negatively correlated with both egg production and fertilization rate, providing additional supportive evidence that the changes observed in egg production and fertilization rate in the present study are a result of hydrocarbon exposure and not natural variation between tanks. Importantly, it has been demonstrated that laboratory sheepshead minnow have higher reproductive potential than wild sheepshead minnow and may underestimate the ecological effects on wild fish (Rutter et al. 2012). It is possible that the reproductive effects reported in the present study of laboratory sheepshead minnow may have been even more severe in wild populations of sheepshead minnow that experienced oil exposure in the Gulf of Mexico.

Although low HEWAF exposure increased F_0 egg production, the eggs that were produced experienced developmental deficits, which may affect survival in a natural habitat. The biphasic dose–response pattern ("hormesis") observed in F_0 egg production in the present study

is a well-documented phenomenon in toxicology (Calabrese et al. 2007) and has been demonstrated in fish reproduction studies (Pawlowski et al. 2004; Tilton et al. 2005; Bosker et al. 2010; van den Heuvel et al. 2010). High variability between tanks lowered statistical power and limited interpretation of F_1 egg production data. Although there is no consensus on critical effect size for fish reproductive tests, a commonly used size is 25%, indicating that a 25% change in egg production would cause adverse effects on the fish population (Bosker et al. 2009; Munkittrick et al. 2009). This suggests that the changes observed in egg production in the present study, although not statistically significant, could be biologically significant and lead to population-level consequences in sheepshead minnow.

The present study demonstrated an array of transgenerational effects, including F_1 developmental changes and impaired F_2 prey capture ability. Other studies have demonstrated developmental effects in fish embryos and larvae exposed to PAHs (White et al. 1999; Incardona et al. 2004; Booc et al. 2014), including reduced hatch frequency, decreased survival, and cardiac abnormalities. A study investigating the developmental effects of PAH exposure on zebrafish embryos found that phenanthrene, a 3-ring PAH, caused cardiac dysfunction, including bradycardia and arrhythmias, and pyrene, a 4-ring PAH, caused reduced blood circulation, anemia, and neuronal cell death (Incardona et al. 2004). Embryonic cardiac effects resulting from hydrocarbon exposure, as demonstrated in the F_1 embryos from sheepshead minnow exposed to low HEWAF in the present study, is a well-established phenomenon in fish. It has been demonstrated that PAHs can cause altered cardiac functioning directly, in PAH-exposed embryos (Incardona et al. 2004, 2009, 2014; Bosker et al. 2017), and indirectly, in embryos collected from PAH-exposed parental fish (Corrales et al. 2014; Perrichon et al. 2015).

Unlike the other developmental endpoints in the present study that measured morphological alterations, prey capture is a behavioral endpoint that requires coordination of several physiological systems, making it a highly sensitive sublethal endpoint to assay. The 2 time points reported for this assay, 2 and 5 min, may simulate environmental situations of limited and abundant prey availability, respectively. Diminished prey capture ability, demonstrated in the F_1 and F_2 generations from high HEWAF-exposed F_0, could impair growth, delay development, increase risk of predation, and decrease survivorship (Zhou et al. 2001). Although the mechanism of these transgenerational effects of hydrocarbons in fish is unclear, it is possible that epigenetics may play a role because Fang et al. (2010) reported that PAH exposure may alter DNA methylation patterns in mummichog embryos.

In adult F_0 sheepshead minnow exposed to high HEWAF, LSI values were significantly increased and positively correlated with body burdens of parent and alkyl PAHs. Increased LSI has been demonstrated to be a physiologic response to hydrocarbon exposure in fish and is thought to be a hyperplastic or hypertrophic response to increase capacity to metabolize hydrocarbons (Heath 2018).

In conclusion, the present study demonstrated that short-term (14-d) exposure of sheepshead minnow to an environmentally relevant mixture of PAHs (HEWAF) resulted in a range of somatic and reproductive effects in the exposed F_0 generation as well as developmental effects in the F_1 and F_2 generations. The F_1 generation was maintained through the adult phase and observed to have reproductive effects that, though not statistically significant, may be of biological significance. These data suggest that HEWAF exposure can cause a range of physiological effects in exposed adult fish, in addition to transgenerational effects in offspring without additional HEWAF exposure, both of which could have population-level consequences.

Correlations between tissue PAHs and observed endpoints provide evidence that the physiological effects observed were caused by hydrocarbon exposure. These data demonstrate that oil from the Deepwater Horizon oil spill was capable of causing significant effects in exposed sheepshead minnow.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4340.

Data Accessibility—Data are publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative at https://data.gulfresearchinitiative.org/data/R2.x213.000:0003 (DOI: 10.7266/N74M92WR).

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Figure 1. Effect of high-energy water accommodated fraction (HEWAF) exposure on reproductive endpoints in F_0 sheepshead minnow. (**A**) Average egg production per female per day during the pre-exposure (10 d) and exposure (14 d) periods. Different letters indicate significant differences within the pre-exposure and exposure periods, using one-way analysis of variance (ANOVA) with the Holm-Sidak test ($p < 0.05$). (**B**) Cumulative egg production per female during exposure to HEWAF. Different letters indicate significant differences at the end of the exposure period, using one-way ANOVA with the Holm-Sidak test ($p < 0.05$). (**C**) Fertilization success of eggs collected from tanks on days 7 and 10 of exposure. Fertilization rate of embryos from high HEWAF exposure was significantly lower than that from low HEWAF exposure. Different letters indicate significant differences within the collection day, using 2-way ANOVA with the Holm-Sidak method ($p < 0.05$). (**A–C**) All data are expressed as mean ±

standard error, $n = 6$ tanks per treatment. Low = low HEWAF (1.7 ng/mL total PAHs); High = high HEWAF (17 ng/mL total PAHs).

Figure 2. Effect of high-energy water accommodated fraction (HEWAF) exposure on developmental endpoints of F_1 sheepshead minnow. All data are expressed as mean ± standard error and were analyzed by 2-way analysis of variance with Holm-Sidak test ($p < 0.05$). Different letters indicate significant differences within factors. Significant main effects and interactions are described in *Results*. (**A**) Embryo heart rates measured 2 d postcollection. (**B**) Hatch success of embryos. (**C**) Average time to hatch of embryos. (**D**) Embryo length at hatch. (**E**) Length of larvae 10 d posthatch (dph). Low = low HEWAF (1.7 ng/mL total PAHs); High = high HEWAF (17 ng/mL total PAHs).

Figure 3. Effect of high-energy water accommodated fraction (HEWAF) exposure on prey (*Artemia salina*) capture ability of F_1 sheepshead minnow, measured at 10 d posthatch for 2 min (**A**) and 5 min (**B**). All data are expressed as mean ± standard error and were analyzed by 2-way analysis of variance with the Holm-Sidak test ($p < 0.05$). Different letters indicate significant differences within factors. Significant main effects and interactions are described in *Results*. Low = low HEWAF (1.7 ng/mL total PAHs); High = high HEWAF (17 ng/mL total PAHs).

Figure 4. Effect of F_0 high-energy water accommodated fraction (HEWAF) exposure on cumulative egg production of F_1 sheepshead minnow. Cumulative egg production per female during the 14-d reproductive test. Data are expressed as mean ± standard error. Letters indicate no significant differences using one-way analysis of variance ($p = 0.521$). Sample sizes are $n = 7$ tanks for control and low HEWAF and $n = 6$ tanks for high HEWAF. Low = low HEWAF (1.7 ng/mL total PAHs); High = high HEWAF (17 ng/mL total PAHs).

Figure 5. Effect of F_0 high-energy water accommodated fraction (HEWAF) exposure on prey (*Artemia salina*) capture ability of F_2 sheepshead minnow, measured at 10 d posthatch for 2 and

5 min. Different letters indicate statistical significance using one-way analysis of variance on ranks with Dunn's test ($p < 0.05$) within each time point. Low = low HEWAF (1.7 ng/mL total PAHs); High = high HEWAF (17 ng/mL total PAHs).

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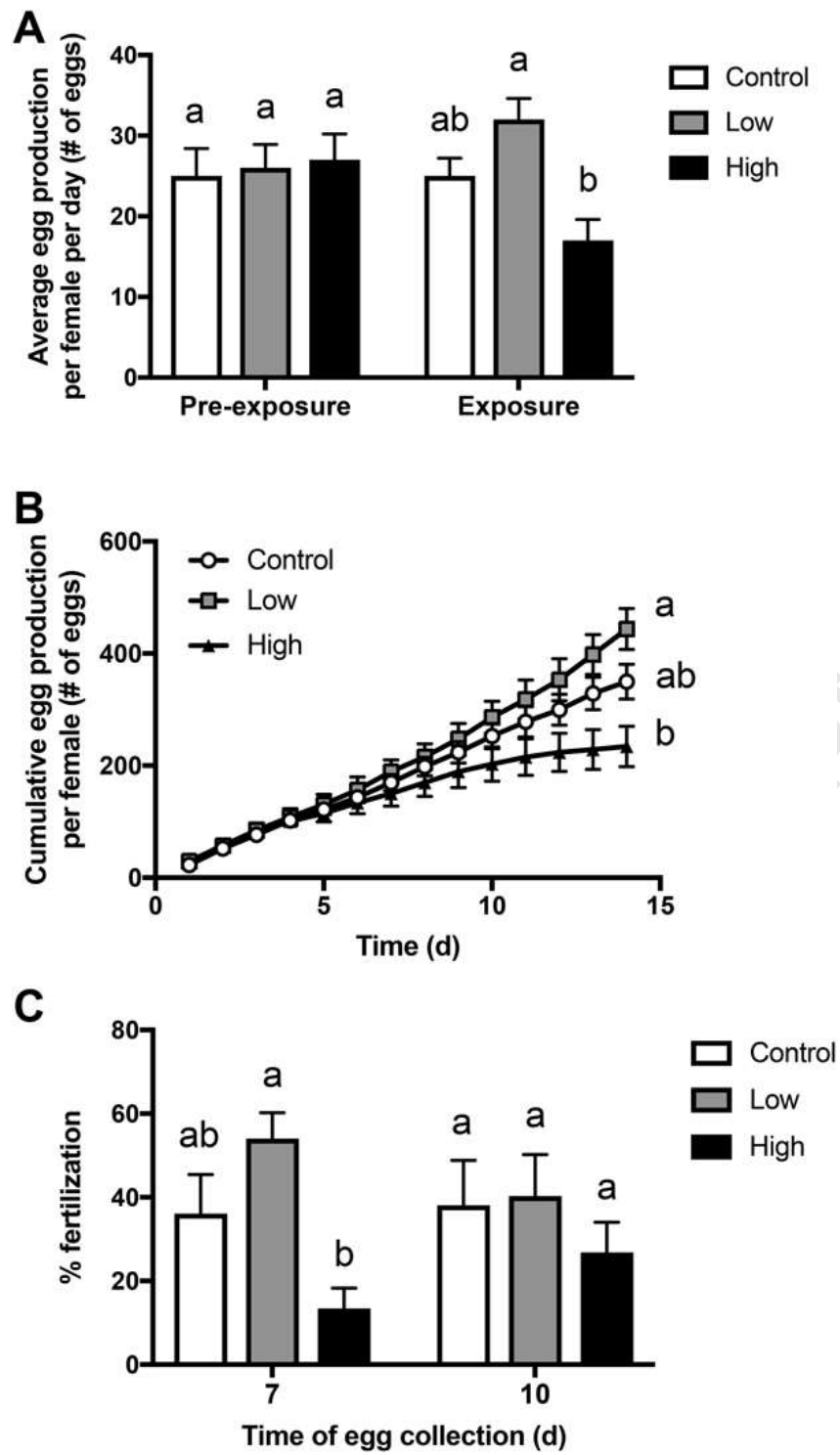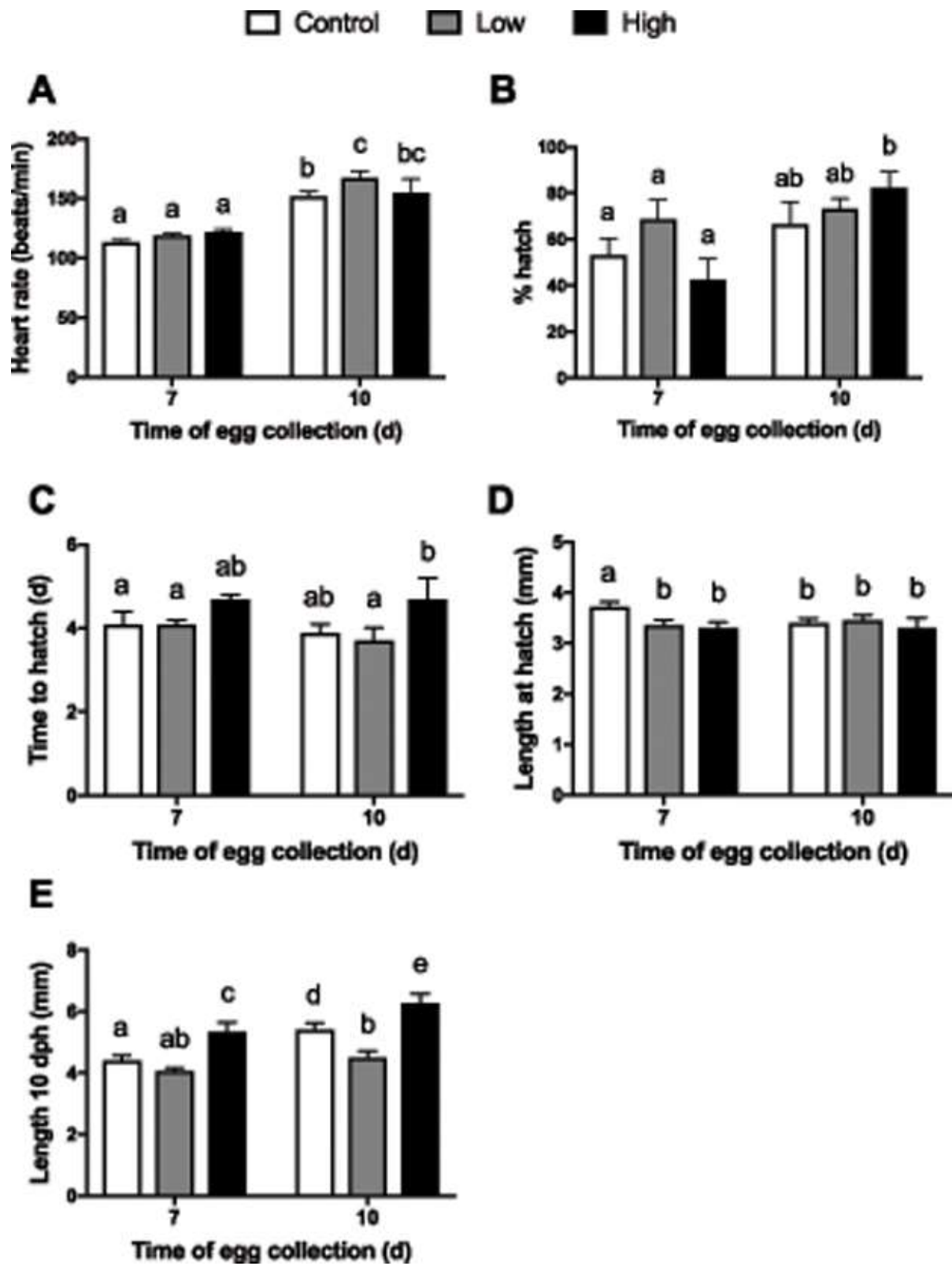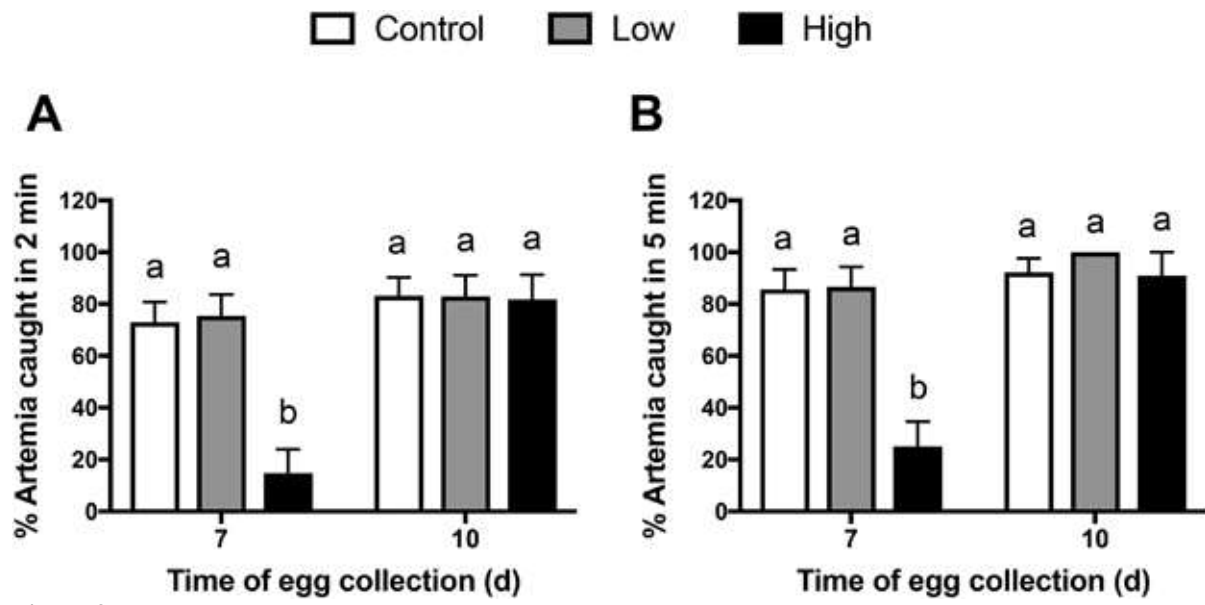
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