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Effects of *Deepwater Horizon* crude oil exposure, temperature and developmental stage on oxygen consumption of embryonic and larval mahi-mahi (*Coryphaena hippurus*)

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ABSTRACT

The timing and location of the 2010 *Deepwater Horizon* (DWH) incident within the Gulf of Mexico resulted in crude oil exposure of many commercially and ecologically important fish species, such as mahi-mahi (*Coryphaena hippurus*), during the sensitive early life stages. Previous research has shown that oil exposure during the embryonic stage of predatory pelagic fish reduces cardiac function – a particularly important trait for fast-swimming predators with high aerobic demands. However, it is unclear whether reductions in cardiac function translate to impacts on oxygen consumption in these developing embryos and larvae. A 24-channel optical-fluorescence oxygen-sensing system for high-throughput respiration measurements was used to investigate the effects of oil exposure, temperature and developmental stage on oxygen consumption rates in embryonic and larval mahi-mahi. Oil-exposed developing mahi-mahi displayed increased oxygen consumption, despite clear cardiac deformities and bradycardia, confirming oxygen uptake and delivery from a source other than the circulatory system. In addition to metabolic rate measurements, nitrogenous waste excretion was measured to test the hypothesis that increased energy demand was fueled by protein catabolism. This is the first study to our knowledge that demonstrates increased energy demand and energy depletion in oil-exposed developing mahi-mahi.

1. Introduction

The *Deepwater Horizon* (DWH) incident was the largest marine oil spill to occur in U.S. history, and resulted in the release of over 3 million barrels of crude oil into the northern Gulf of Mexico (GoM) during the spring and summer of 2010 (Crone and Tolstoy, 2010; McNutt et al., 2011; Sun et al., 2015; PDARP, 2016). The timing and location of this spill coincided with the temporal spawning window of many ecologically and economically important pelagic fish species (Block et al., 2005; Brown-Peterson et al., 2001; Rooker et al., 2012, 2013), including mahi-mahi (Gibbs and Collette, 1959; Palko et al., 1982). It has been well established that developing fish embryos are especially vulnerable to the toxicity of crude oil-derived polycyclic aromatic hydrocarbons (PAHs), which cause a suite of cardiovascular and morphological abnormalities (Dubansky et al., 2013; Huang et al., 2012; Incardona et al., 2004, 2009, 2011; Zhang et al., 2012).

PAH toxicity is structure-dependent, and thus many studies have focused on how factors such as weathering, use of dispersants and microbial action affect the chemical composition of oil mixtures (Carls and Meador, 2009; Esbaugh et al., 2016; Lehr et al., 2010; Stegeman and Lech, 1991). Weathering tends to remove lower molecular weight PAHs from oil mixtures through evaporation, subsequently producing oil slicks that are dominated by the tricyclic fluorenes, dibenzothiophenes, and phenanthrenes (Carls et al., 1999; Heintz et al., 1999; Short and Heintz, 1997). One unique aspect of the DWH spill was that it originated at depth and under high energy, facilitating greater dissolution of lower molecular weight PAHs into the water column during the ascent to surface waters, and thus generating oil slicks with an increased ratio of dissolved-phase PAHs with higher molecular weights and lower solubility (Reddy et al., 2012; Ryerson et al., 2012). Gulf of Mexico pelagic species, such as mahi-mahi, typically produce small and buoyant embryos that develop rapidly and hatch into buoyant larvae (Ditty et al., 1994). These pelagic embryos that float in the upper layers of the water column are likely directly exposed to PAHs in

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surface oil slicks, along with multiple concurrent stressors, such as UV-radiation and temperature stress.

Different PAH compounds have been found to produce distinct effects on the sensitive early life stages (ELS) of developing fish; studies indicate that 3-ringed (tricyclic) PAHs are primarily responsible for causing embryonic cardiac abnormalities and functional defects including bradycardia, arrhythmias and heart failure (Esbaugh et al., 2016; Incardona et al., 2009, 2014). This loss of cardiac function is implicated in causing additional developmental sequelae, such as impaired eye and jaw formation that may hinder survival by reducing the ability of free-swimming larvae to catch prey following yolk depletion (Incardona et al., 2004). Such structural defects may arise due to a combination of reduced vascular pressure limiting perfusion and angiogenesis resulting from the decreased circulation associated with impaired heart function. Thus, to better understand the significance of potential limitations to oxygen delivery arising from ELS crude oil exposure, where oxygen demand may be elevated due to PAH effects and detoxification, one main objective of this study was to explore the effects of exposure to a weathered slick oil obtained from the GoM during the DWH oil spill on the oxygen consumption and energy utilization of developing mahi-mahi. This information may also help to better understand the extent of ELS loss from oiled spawning habitats – an important question for fisheries management and conservation.

In the present study, we tested the hypothesis that oil-exposed mahi-mahi embryos and larvae may not yet display reduced oxygen consumption due to limitations in oxygen uptake and delivery resulting from cardiac abnormalities, because of their small size and incomplete development of a fully functioning circulatory system. The larvae used for these experiments were recently hatched (~50–60-hpf) and probably rely more heavily on cutaneous respiration or simple diffusion for oxygen uptake. Fish larvae are generally considered to meet O_2 demands by simple diffusion, as the average body radius of many larval fish including mahi-mahi larvae fall under the 1 mm diffusion threshold that limits sufficient oxygen supply to these developing fish (average mahi-mahi larvae radius is 0.4 mm, pers. obs.) (Hill et al., 2004). At this early life stage, the developing fish heart may have numerous other functions, such as angiogenesis, nutrient delivery and waste elimination and thus structural cardiac impairments still likely produce alternative negative impacts at this stage.

Surface slick oil from the DWH spill was prepared as a high-energy-water-accommodated fraction (HEWAF) to generate environmentally relevant PAH concentrations and compositional profiles. Embryos were, therefore, exposed to oil mixtures rich in tricyclic PAHs, known to be highly cardiotoxic. Our first hypothesis was proven half true, as oxygen consumption in larvae exposed to oil for 24-hpf (hours post fertilization) was not limited by a compromised circulatory system; oil-exposed larvae actually exhibited significantly higher rates of oxygen consumption compared to control larvae. These results suggest that metabolically costly processes associated with PAH exposure, such as detoxification mechanisms, may be raising the metabolic demand of these developing mahi-mahi. This led to a second hypothesis: increased metabolic demand in oil-exposed, developing mahi-mahi is fueled by protein catabolism. To test this hypothesis, ammonia, urea and total nitrogenous waste excretion were measured in embryonic and larval mahi-mahi exposed to oil for 24-hpf.

To better understand how increased metabolic demand associated with PAH exposure may affect embryos and larvae in the wild, we explored the potential synergistic impacts of elevated temperature and oil exposure on the oxygen consumption and nitrogenous waste excretion in developing mahi-mahi. We hypothesized that elevated temperature may increase toxicity by increasing basal metabolic demand further, leaving limited energetic reserves for PAH metabolism and detoxification. Also stemming from the observations of elevated metabolic rates in oil-exposed developing mahi-mahi, was a prediction of corresponding increases in energy depletion by oil-exposed embryos. The prediction on energy depletion was tested by measurements of yolk sac depletion in embryonic mahi-mahi. Endogenous yolk sacs provide the sole substrates for energy in most developing fish embryos; and both the rate of yolk absorption and the efficiency of yolk utilization are important factors determining embryonic growth and survival (Heming and Buddington, 1988). The reported results are the first to show increased energy demand and suggest energy depletion in oil-exposed fish embryos and larvae.

2. Methods

2.1. Experimental animals

Mahi-mahi (*Coryphaena hippurus*) broodstock were caught off the coast of South Florida using hook and line angling techniques and then directly transferred to University of Miami Experimental Hatchery (UMEH). Broodstock were acclimated in 80 m^3 fiberglass maturation tanks equipped with recirculating aquaculture systems allowing for water temperature control. All embryos used in the experiments described herein were collected within 2–10 h following a volitional (non-induced) spawn using standard UMEH methods (Benetti et al., 2008; Stieglitz et al., 2012). A prophylactic formalin treatment (37% formaldehyde solution) was administered to the embryos (100 ppm for 1 h), followed by 30 min of flushing with a minimum of 300% water volume in the treatment vessel using filtered, UV-sterilized seawater. A small sample of eggs was collected from each spawn to microscopically assess fertilization rate and embryo quality. Spawns demonstrating low fertilization rate (<85%) or frequent developmental abnormalities (>5%) were not used.

2.2. Preparation of water accommodated fractions

The oil from the surface (referred to herein as OFS) used to prepare all HEWAFs (high-energy water-accommodated fraction) was collected via skimming operations from surface waters by British Petroleum for testing purposes, and subsequently transferred under chain of custody to the University of Miami (sample ID: OFS-20100719-Juniper-001 A0091G & A00919). Each HEWAF was prepared on the day of use at a loading rate of 1 g of oil per liter of 1 μm filtered, UV-sterilized seawater and mixed in a Waring CB15 blender (Torrington, CT) at low speed for 30 s. The mixture was immediately transferred to a glass separatory funnel and left to settle for 1 h. The lower 90% of the mixture was then carefully drained and reserved for subsequent use as 100% WAF (unfiltered) that was diluted to nominal concentrations of 2, 4 or 8% using UV-sterilized seawater.

2.3. Embryo exposures

Embryos were transported from UMEH to RSMAS around 3–6-hpf and immediately transferred to UV-sterilized seawater for controls or either 2, 4 or 8% HEWAF dilutions for treatment exposures. Exposures took place in 1 L glass beakers (~30 embryos per beaker) and lasted for 24 h. After the 24-h exposure, both control and treatment embryos were transferred to clean 1 L beakers with fresh UV-sterilized seawater for the duration of the experiment (Supplementary Fig. 1). Aeration was not used, to reduce PAH depletion over the 24-h exposure period. Dissolved oxygen was measured daily to ensure levels did not drop below that which would be considered stressful to developing embryos (~80% air saturation). Exposures and grow-out periods were conducted in a

temperature and light-controlled (photoperiod: 16 L:8 D) environmental chamber. To test the combined effects of oil exposure and temperature, developing mahi-mahi were exposed and raised at either 26 °C (ambient) or 30 °C. Exposures at 30 °C included only 2% and 4% HEWAF dilutions, as an 8% HEWAF dilution combined with high temperature was too stressful for the developing embryos and produced very high mortality. Due to the large number of individuals required for oxygen consumption and nitrogenous waste excretion measurements, 2–3 trials were conducted at each exposure dilution, and thus numerous cohorts of mahi-mahi were tested for this experiment. Batches of embryos in these experiments were collected from one of two tanks containing one male and multiple females. Oil- exposed mahi-mahi were only compared to controls from the same cohort of embryos within a given trial to reduce confounding variables resulting from batch effects (see Discussion 4.1).

2.4. Water chemistry analysis

Samples for total sum PAH (ΣPAH) analysis were collected immediately before and immediately after the 24-h exposure period for each trial. Samples were also taken from the control beakers at the start of the exposure period, and from the treatment beakers after transfer to clean UV-sterilized seawater, to ensure there was no background level or transfer of PAHs. Each sample was collected into a 250 mL amber glass bottle with no head space and immediately stored at 4 °C. Sample bottles were shipped overnight on ice to ALS Environmental (Kelso, WA) for analysis by gas chromatography/mass spectrometry- selective ion monitoring (GC/MS-SIM; based on EPA method 8270D). Reported ΣPAH values represent the sum of 50 PAH analytes, selected by the EPA based on individual toxicity and concentration and are presented as geometric means of initial and final measurements.

In addition to ΣPAH analysis, the following water parameters were measured daily to ensure water quality: temperature, pH, dissolved oxygen (DO), salinity, and total ammonia. Temperature and DO were measured using a ProODO hand held optical DO probe and meter (YSI, Inc., Yellow Springs, OH) and pH was measured using a PHM201m (Radiometer, Copenhagen, Denmark) fitted with a combination glass electrode. The pH and DO probes were calibrated daily. Salinity was measured using a refractometer. Samples for measurements of total ammonia were collected daily, excluding the first day, to confirm ammonia accumulation did not exceed levels that might be stressful to developing mahi-mahi. Total ammonia was determined using a micro-modified colorimetric assay (Ivančič and Degobbis, 1984).

2.5. Oxygen consumption trials

A 24-channel optical fluorescence, oxygen-sensing system (Loligo Systems, Copenhagen, Denmark) was used to test the singular and combined effects of oil exposure and temperature on the oxygen consumption of developing mahi-mahi embryos and larvae. The microplate sensor dish reader (SDR) is a system with integrated sensors in 24 separate wells. Two embryo oxygen consumption trials were conducted for each different dilution and temperature combination. The first embryo trial started immediately following the 24-h exposure period (~30-hpf), while the second trial was set-up later in the day before hatching (~34-hpf) to explore possible delayed effects later in development. In the following, the two embryo oxygen consumption trials will be referred to as E1 and E2. Mahi-mahi embryos typically hatch ~36-hpf under water temperatures of ~26 °C, and thus larvae oxygen consumption trials (L) were conducted on the following day (~50-hpf).

Briefly, the set-up of the microplate SDR system was as follows: embryos or larvae were added to individual wells (125, 200 or 650 μL) as quickly as possible, after which the microplate was carefully placed in a temperature-controlled flow-through water bath. A silicon membrane covered in parafilm was then placed on top of the microplate, effectively creating an oxygen impermeable seal on each individual well. The water bath was closed tightly and then placed on top of an SDR reader and into a temperature-controlled incubator. The flow-through water bath was used to ensure temperature consistency throughout the duration of the experiment. Control wells (3–4) were filled with seawater but no organism, to test and correct for any background level of respiration. For analysis, oxygen consumption by time was plotted for individual wells. The first 10–20% (pO_2 in% air saturation) of slope regions showing linear decreases in oxygen were used to calculate oxygen consumption over time. Any portion of the slope that dipped below 70% air saturation was not used for analysis. Last, the data were corrected for controls, volume of seawater and number of individuals per well, and expressed as picomoles individual^{-1} min^{-1}.

2.6. Nitrogenous waste excretion trials

Nitrogenous waste excretion experiments were conducted on embryonic and larval mahi-mahi to test the hypothesis that increased metabolic demand in oil-exposed individuals is fueled by protein catabolism. Either 40 embryos or 10 larvae were added to individual wells with 2 mL of UV-sterilized seawater each, on a 12-well plate. Control wells filled with seawater but no organisms were used to correct for any background levels of ammonia or urea. The plate was then put into a temperature-controlled incubator for 7–10 h, after which water samples were collected for future measurements of nitrogenous waste excretion. Micro-modified Indophenol Blue and Diacetyl Monoxime methods were later conducted to test for ammonia (Ivančič and Degobbis, 1984) and urea (Boyde and Rahmatullah, 1980) excretion, respectively. Last, the data were corrected for background ammonia and urea, volume of seawater and number of individuals per well, and expressed as picomoles individual^{-1} min^{-1}.

2.7. Image collection and analysis

Imaging was used to explore the singular and combined effects of oil exposure and temperature on 3 parameters: pericardial and yolk sac area, heart rate and yolk sac depletion. Embryos or larvae were mounted 2–3 at a time over 2% methylcellulose in seawater and imaged using either a Fire-i400 or Fire-i530c digital camera (Unibrain, San Ramon, CA) mounted on a Nikon SMZ800 stereomicroscope. Images and videos were collected on a MacBook laptop using Photo Booth software and calibrated using a stage micrometer. A heating/cooling temperature controller and thermal stage (Brook Industries, Lake Villa, IL) were used to ensure images and videos were collected at the same temperature used to raise the developing mahi-mahi in a given trial.

Pericardial and yolk sac area was measured as a proxy for the extent of edema present in yolk sac larvae (~50-hpf). Edema was observed as fluid accumulation sufficient to distort the normal smooth bullet shape of the anterior yolk mass, and occurred in the form of a concave or pointed wedge shape of the anterior yolk mass. Pericardial and yolk sac area was measured using ImageJ version 1.46r (rsbweb.nih.gov/ij/) from a perimeter drawn with the freehand tool enclosing the area. Lines were drawn around the perimeter of the pericardial area and yolk sac up to the point of the oil globule and then area was calculated. Lines were drawn across the boundary of the yolk sac only if distortion of the yolk mass was clearly evident due to fluid accumulation, usually indicated by a sharp dark line (Fig. 6, panel 3). Heart rate was determined in yolk sac larvae (~50-hpf) by simply counting the number of heartbeats in a given video clip (~20 s) played back at half speed.

Finally, yolk sac depletion was measured in mahi-mahi embryos (~30-hpf) raised at 30 °C only. Depletion of yolk sacs in oil-exposed embryos became apparent at higher temperatures, and thus this measurement was added into the experiment. Yolk sac depletion area was measured as a ratio of yolk sac area over total embryo area, again using ImageJ.

2.8. Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Differences were tested for statistical significance using SigmaPlot 13.0 (Systat Software, Inc., San Jose, CA). All tests were done using a two-way ANOVA, followed by Tukey multiple comparison procedure, unless stated otherwise. Details of these statistical tests are presented in the figure legends. Differences between means were deemed significant at $P < 0.05$.

3. Results

3.1. PAH concentrations and composition of HEWAF preparations

Depletion of ΣPAH concentrations and shifts in chemical composition occurred in all of the HEWAF dilutions over the 24-h exposure, as was to be expected due to known natural depletion of PAHs over time. All biological data is reported as a function of the geometric mean of the initial and final ΣPAH concentrations for the relevant nominal concentrations. The percent distribution of individual PAHs was similar among all oil exposures, with the tricyclic fluorenes, dibenzothiophenes, and phenanthrenes/anthracenes predominating (Supplementary Fig. 2). Nominal concentrations of HEWAF produced nearly proportional changes in ΣPAHs, although this relationship weakened at the lowest concentration (2%), which had the greatest variability in ΣPAH concentrations on a trial-to-trial basis.

3.2. Oxygen consumption trials

Control and oil-exposed embryos raised at 26 °C exhibited no difference in oxygen consumption at any of the exposure concentrations. However, larvae exposed to 21.8 or 49.6 μg L^{-1} ΣPAH as embryos displayed significantly greater oxygen consumption than controls (Fig. 1) with a trend suggesting increased rates at higher exposure concentrations (Fig. 2B).

Embryos raised at 30 °C and exposed to 15.2 or 30.4 μg L^{-1} ΣPAH had significantly greater oxygen consumption than controls at the E1 stage; however, there was no significant differences at the E2 or L stages for either concentration (Figs. 3 and 4). Exposures to a higher concentration (corresponding to 8% HEWAF) were performed but resulted in high mortality and thus this trial was removed from the dataset.

To explore temperature effects, control oxygen consumption values were pooled among trials for all three developmental stages performed at 26 and 30 °C. As expected, developing mahi-mahi at the E2 and L stage raised at 30 °C displayed significantly greater oxygen consumption than individuals raised at 26 °C.

3.3. Nitrogenous waste excretion trials

There were no significant differences in nitrogenous waste excretion between control and oil-exposed embryos raised at 26 °C at any of the HEWAF concentrations applied (Supplementary Fig. 3). Oil exposure had a significant effect on urea excretion, but no effect on ammonia or total nitrogenous waste excretion in larvae raised at 26 °C. The post hoc Tukey test revealed larvae exposed to 6 or 44.1 μg L^{-1} ΣPAH for 24-h as embryos had significantly greater urea excretion compared to controls (Fig. 5A).

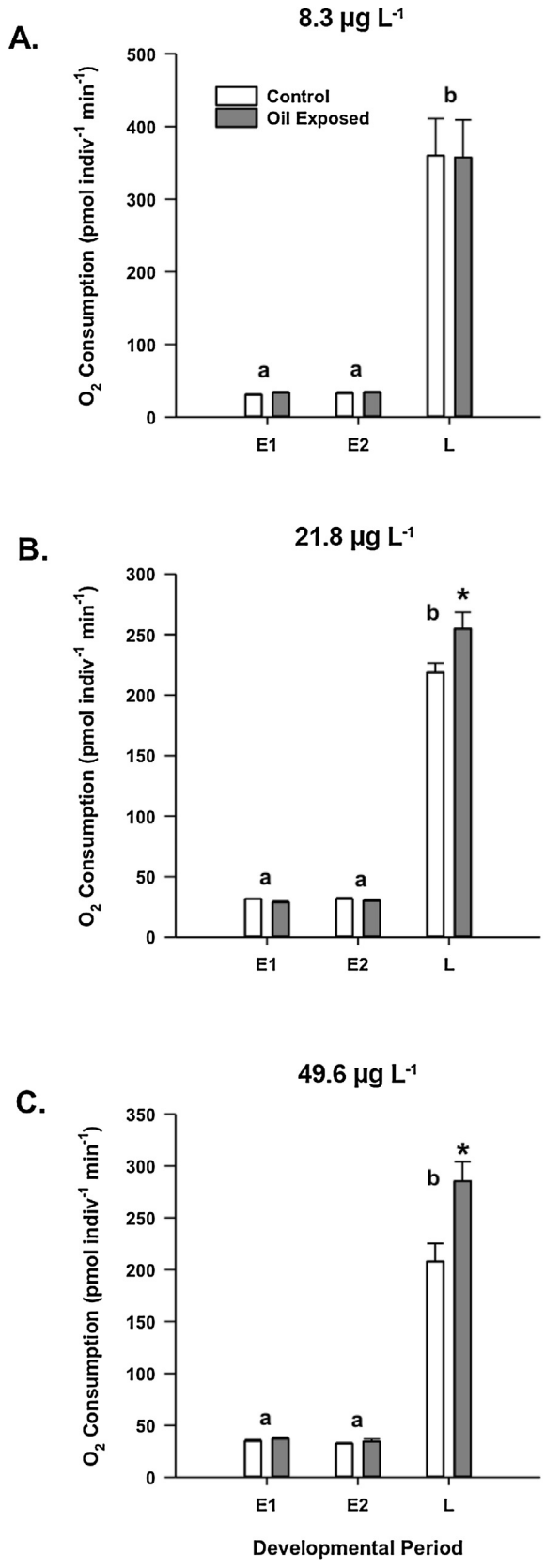

Fig. 1. Oxygen consumption measurements in developing mahi-mahi raised at 26 °C and exposed to control (0.04), 8.3 (panel A), 21.8 (panel B) or 49.6 (panel C) μg L^{-1} ΣPAHs for 24-hpf. There were 8–10 replicates of either 2 or 4 embryos or 1 larva per well. Data are presented as mean ± SEM. Two-way ANOVAs using developmental stage and oil exposure concentration as factors were used to determine significant differences in oxygen consumption in developing mahi-mahi.

Oil exposure had a significant effect on ammonia and total nitrogenous waste excretion, but not urea excretion in larvae raised at 30 °C. The post hoc Tukey test revealed larvae exposed to 26.6 μg L^{-1} ΣPAH for 24-h as embryos exhibited significantly higher ammonia and total nitrogenous waste excretion compared to controls (Fig. 5B).

3.4. Sublethal cardiotoxicity tests

3.4.1. Pericardial and yolk sac area

Pericardial and yolk sac area was measured as a proxy for pericardial edema in mahi-mahi larvae. Yolk sac larvae raised at 26 °C and exposed to oil for 24-h as embryos displayed significantly greater edema compared to controls only at the highest HEWAF concentration tested (44.1 μg L^{-1} ΣPAH) (Fig. 6A), while larvae raised at 30 °C displayed significantly greater edema at both HEWAF concentrations tested (14.9 and 26.6 μg L^{-1} ΣPAH) (Fig. 6B). Pericardial and yolk sac area was significantly higher in control yolk sac larvae raised at 26 °C compared to individuals raised at 30 °C (Kruskal-Wallis one-way ANOVA).

3.4.2. Heart rate

Heart rate (BPM) was significantly lower in yolk sac larvae raised at 26 °C and exposed to all 3 HEWAF concentrations (6, 18.2 and 44.1 μg L^{-1} ΣPAH) for 24-h as embryos (Fig. 7A). Yolk sac larvae raised at 30 °C also exhibited significantly lower heart rates at both HEWAF concentrations (14.9 and 26.6 μg L^{-1} ΣPAH) (Fig. 7B). Control yolk sac larvae raised at 26 and 30 °C had similar average heart rates (~200 bpm).

3.4.3. Yolk sac depletion

Embryos raised at 30 °C and exposed to both HEWAF concentrations (14.9 and 26.6 μg L^{-1} ΣPAH) displayed significantly greater yolk sac depletion compared to controls (Fig. 8).

4. Discussion

The *DWH* disaster resulted in oil exposure of many large pelagic fish species during the sensitive early life stages. Aside from the acute mortality elicited by this event, additional sublethal effects likely occurred resulting in decreased survival and reduced fitness at later life stages. Assessment of ELS loss is thus both challenging and complex, but also critical when attempting to evaluate overall damage to fish and fisheries caused by the oil spill. To explore these sublethal effects, oxygen consumption and energy utilization were measured in developing mahi-mahi exposed to different ΣPAH for 24-hpf. Oil used in exposures was directly collected from surface waters in the GoM and concentrations chosen have been found to be realistic and ecologically relevant (Bejarano et al., 2013; Diercks et al., 2010; Wade et al., 2011). Results from this study provide new insights into effects of oil exposure on ELS of mahi-mahi.

Significant increases in larval oxygen consumption suggest that oil exposure increased energy demand and metabolism in developing mahi-mahi (Fig. 1). This effect emerged at the larval stage when mahi-mahi were raised at ambient temperatures (26 °C). Embryonic oxygen consumption did not differ from controls. The data also implied a concentration-dependent trend, with a greater increase in larval oxygen consumption after exposure to 49.6 compared to 21.8 μg L^{-1} ΣPAH, and no difference at the lowest exposure (8.3 μg L^{-1}) (Fig. 2).

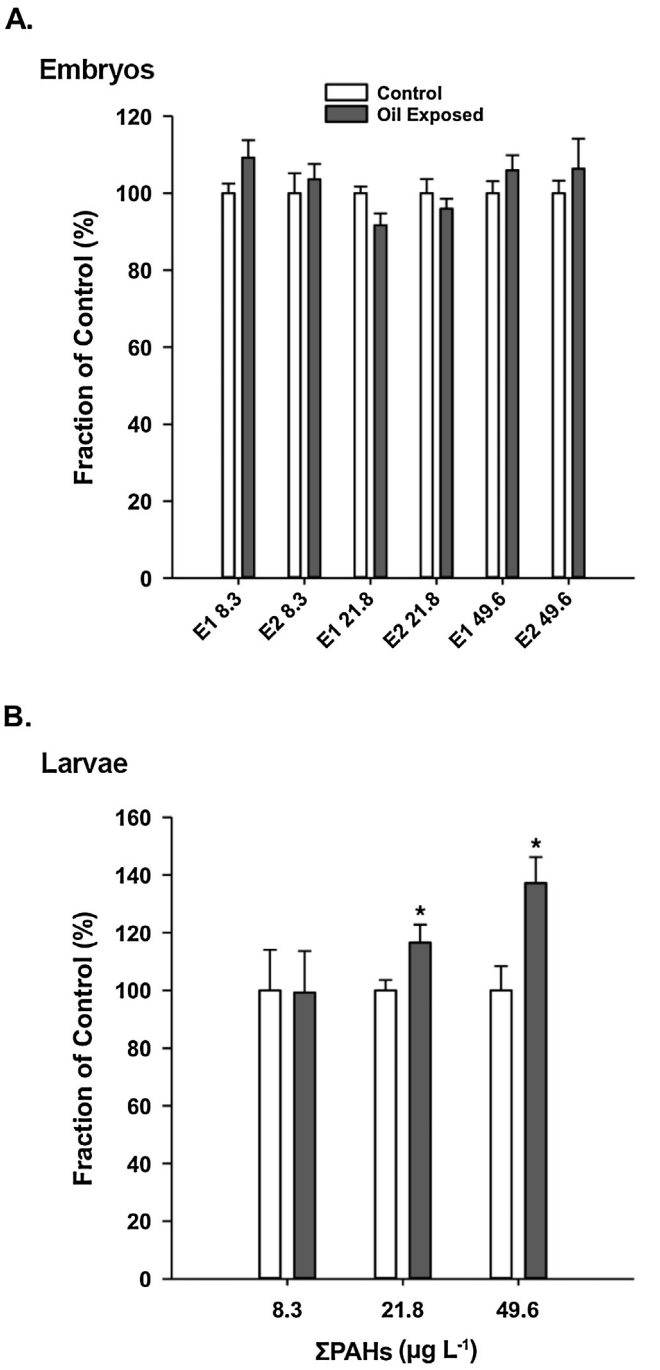

Fig. 2. Oxygen consumption measurements displayed as fraction of the control (%) for embryos (panel A) and larvae (panel B) raised at 26 °C and exposed to 8.3, 21.8 or 49.6 μg L^{-1} ΣPAHs for 24-hpf. There were 8–10 replicates of either 2 or 4 embryos or 1 larva per well. Two-way ANOVAs using developmental stage and oil exposure concentration as factors were used to determine significant differences in oxygen consumption in developing mahi-mahi. *Asterisks* indicate significant differences in oxygen consumption between control and oil-exposed individuals at the same developmental stage.

The effect of oil exposure on energy demand became evident at the earlier, embryonic stages when mahi-mahi were raised at 30 °C. Under ambient conditions, metabolic rate stays relatively stable during embryonic development, up until the point when embryos become negatively buoyant (a couple of hours before hatch), where there is a sharp increase in oxygen consumption. This can be observed when comparing oxygen consumption between controls at the E1 and E2 (prior to hatching) stages (Fig. 3). Although

Different letters indicate significant differences between developmental stages, while *asterisks* indicate significant differences in oxygen consumption between control and oil-exposed individuals at the same developmental stage.

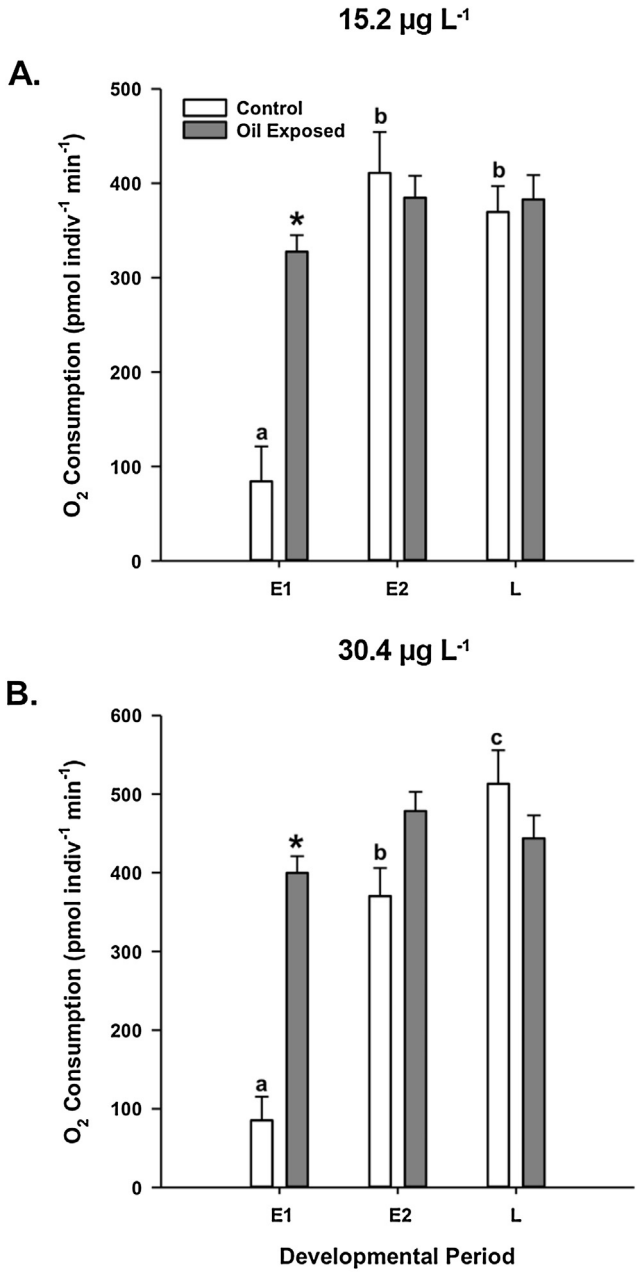

Fig. 3. Oxygen consumption measurements in developing mahi-mahi raised at 30 °C and exposed to control (0.04), 15.2 (panel A) or 30.4 (panel B) µg L^{-1} ΣPAHs for 24-hpf. There were 10–19 replicates of either 2 or 4 embryos or 1 larva per well. Data are presented as mean ± SEM. Two-way ANOVAs using developmental stage and oil exposure concentration as factors were used to determine significant differences in oxygen consumption in developing mahi-mahi. *Different letters* indicate significant differences between developmental stages, while *asterisks* indicate significant differences in oxygen consumption between control and oil-exposed individuals at the same developmental stage.

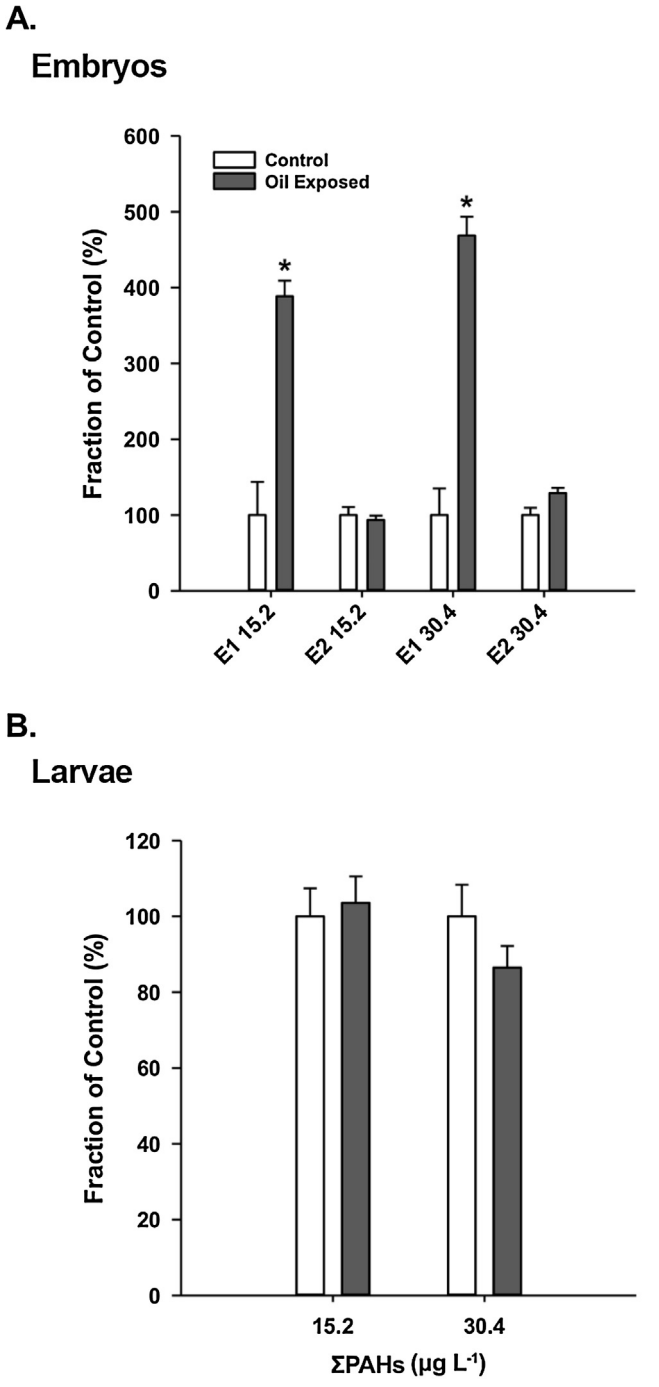

Fig. 4. Oxygen consumption measurements displayed as fraction of the control (%) for embryos (panel A) and larvae (panel B) raised at 30 °C and exposed to 15.2 or 30.4 µg L^{-1} ΣPAHs for 24-hpf. There were 10–19 replicates of either 2 or 4 embryos or 1 larva per well. Two-way ANOVAs using developmental stage and oil exposure concentration as factors were used to determine significant differences in oxygen consumption in developing mahi-mahi. *Asterisks* indicate significant differences in oxygen consumption between control and oil-exposed individuals at the same developmental stage.

little is known about the mechanism used to induce buoyancy change, it is clear that negative buoyancy correlates to hatch time in mahi-mahi embryos raised in ambient, non-stressful conditions (Supplementary Fig. 4).

One of the most surprising outcomes of this study was the observation that oil-exposed embryos raised at higher temperatures became negatively buoyant hours before control embryos at the same temperature, although they hatched at approximately the same time. Thus in oil-exposed embryos, unlike controls, the buoyancy change did not signal impending hatching. In addition to an early onset of negative buoyancy, oil-exposed embryos displayed sustained increased rates of oxygen consumption, only expected during the period directly prior to hatch. Oil-exposed embryos raised at 30 °C exhibited earlier increases in metabolic rate, with oxygen consumption values approximately four or five-fold higher than controls at the E1 stage. At the E2 stage, oxygen consumption values were similar between treatment and controls (Figs. 3 and 4).

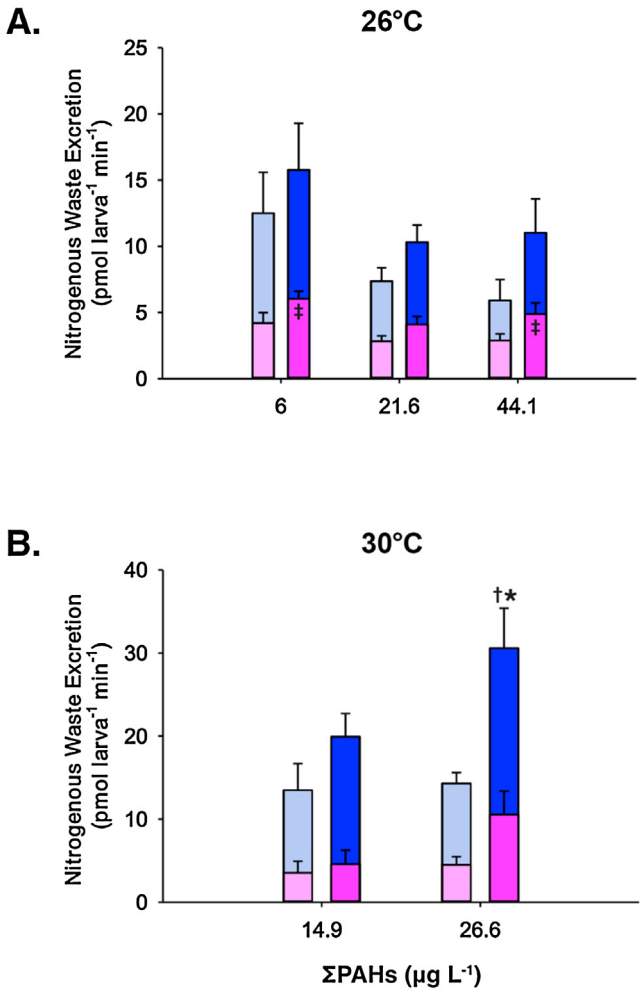

Fig. 5. Total nitrogenous waste excretion, urea (pink) and ammonia (blue) excretion in yolk sac larvae raised at 26 °C (panel A) or 30 °C (panel B). Nitrogenous waste excretion is expressed by light bars in control larvae and by darker bars in oil-exposed larvae. There were 5–8 replicates of either 40 embryos or 10 larvae per well. Data are presented as mean ± SEM. Two-way ANOVAs using developmental stage and oil exposure concentration as factors were used to determine significant differences in nitrogenous waste excretion in developing mahi-mahi. Significant differences between control and oil-exposed larvae are indicated by *asterisks* (total nitrogenous waste), *daggers* (ammonia) and *double daggers* (urea). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

At the time E2 trials were conducted, all embryos in the treatment and control groups were negatively buoyant and thus high metabolic rate was expected due to proximity to hatch time. Therefore, the onset of negative buoyancy has a definitive correlation to increased oxygen consumption rates, although the cause and effect relationship is unclear.

The reason for this premature buoyancy change in oil-exposed embryos is not known. Alterations to the timing of negative buoyancy onset and possible sinking rate could have ecological implications for these developing embryos. Sinking down the water column earlier in development may place them in unfavorable conditions for hatching, post-hatch feeding, and predation risk (Margulies et al., 2007; Stieglitz et al., 2016). Future experiments are needed to further explore the mechanisms and ecological impacts of earlier buoyancy changes and prolonged increases in oxygen consumption. Although non-significant, there was a slight tendency for reduction in oxygen consumption in larvae raised at 30 °C and exposed to the highest oil concentration tested (30.4 µg L^{-1} ΣPAH), possibly indicating a threshold where larval oxygen uptake

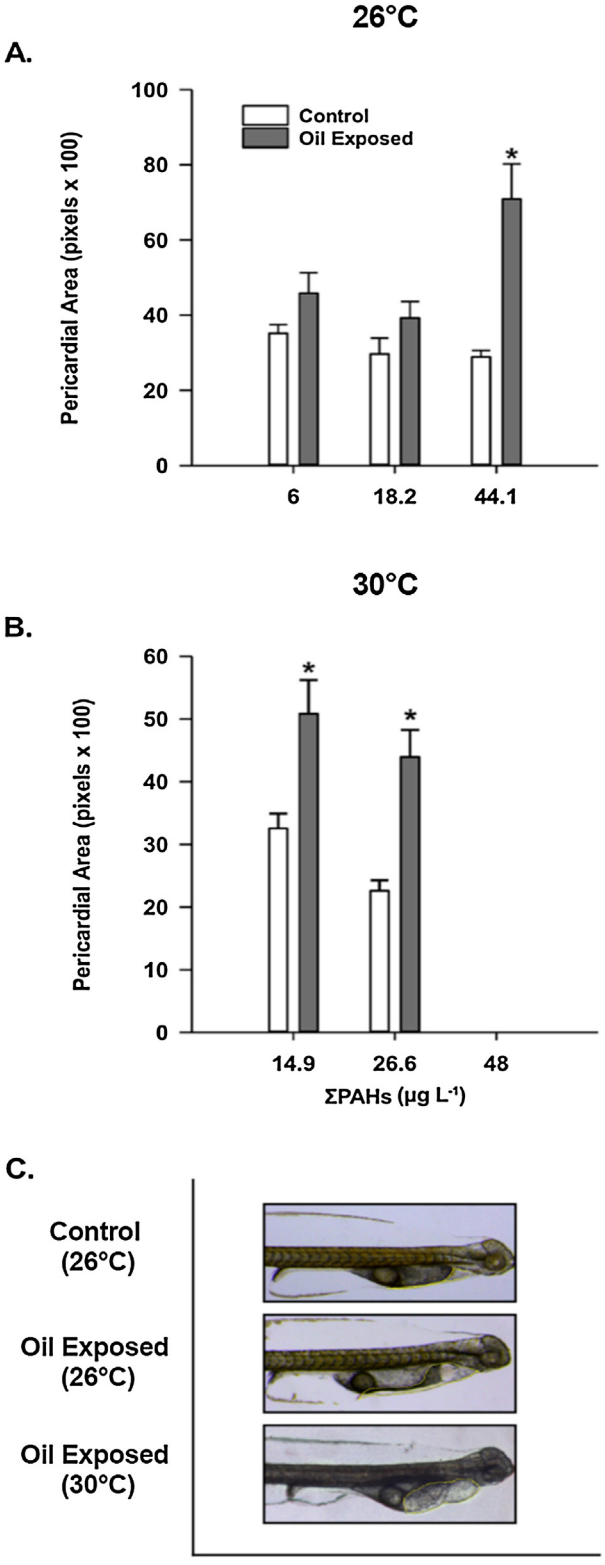

Fig. 6. Pericardial (and yolk sac) area measured in yolk sac larvae raised at 26 °C (panel A) or 30 °C (panel B). The highest concentration exposure (48 µg L^{-1} ΣPAHs) had to be removed from the elevated temperature treatment due to high mortality. Sample sizes range from 24 to 26. Data are presented as mean ± SEM. Two-way ANOVAs using trial (oil exposure concentration) and treatment (control versus oil-exposed) as factors were used to determine significant differences in pericardial area in developing mahi-mahi. *Asterisks* indicate significant differences in pericardial area between control larvae and larvae exposed to different ΣPAHs for 24-hpf. Examples of measurements of pericardial and yolk sac area from larvae raised in UV-sterilized seawater at 26 °C (top), exposed to 4% HEWAF dilution for 24-hpf and raised at 26 °C (middle) and exposed to 4% HEWAF dilution for 24-hpf and raised at 30 °C (bottom) (panel C).

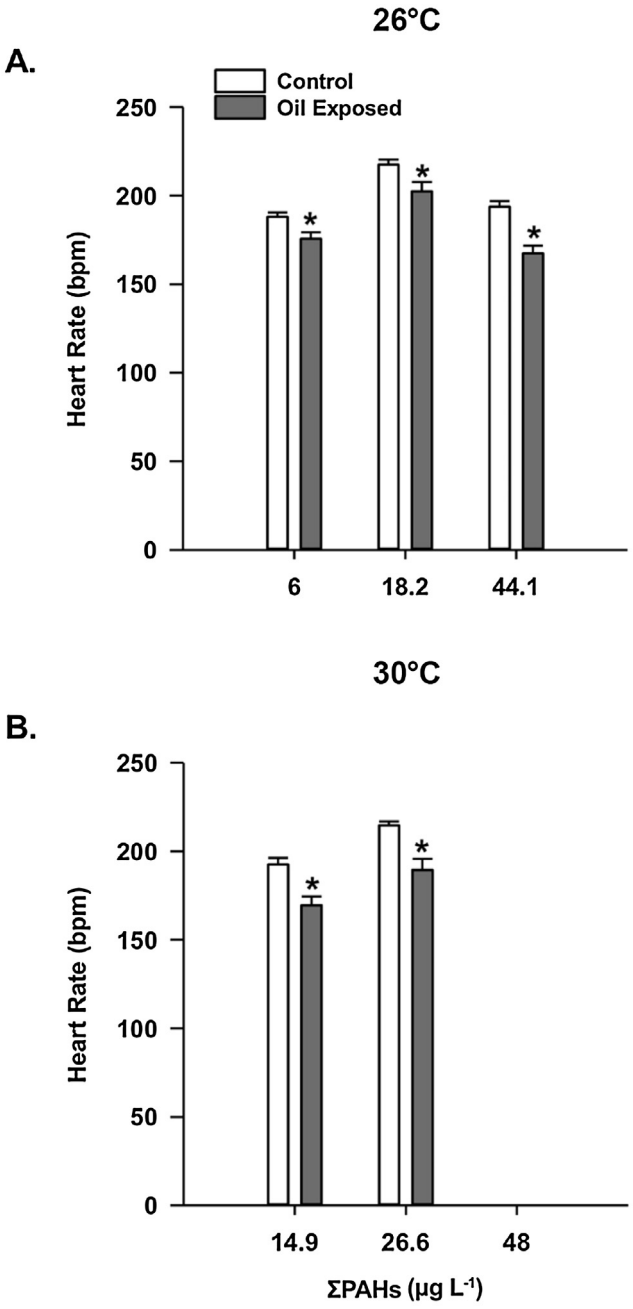

Fig. 7. Heart rate (bpm) measured in yolk sac larvae raised at 26 °C (panel A) or 30 °C (panel B). The highest concentration exposure (48 µg L^{-1} ΣPAHs) had to be removed from the elevated temperature treatment due to high mortality. Sample sizes range from 24 to 26. Data are presented as mean ± SEM. Two-way ANOVAs using trial (oil exposure concentration) and treatment (control versus oil-exposed) as factors were used to determine significant differences in heart rate in developing mahi-mahi. *Asterisks* indicate significant differences in heart rate between control larvae and larvae exposed to different ΣPAHs for 24-hpf.

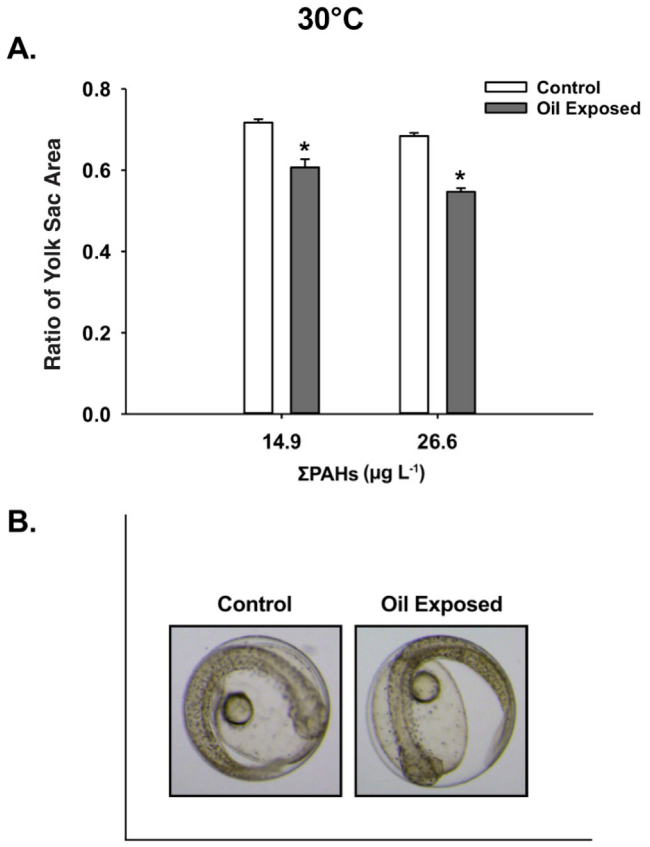

Fig. 8. Ratio of yolk sac depletion area measured in embryos raised at 30 °C and exposed to control (0.04), 14.9 or 26.6 µg L^{-1} ΣPAHs for 24-hpf (panel A). Sample size was 26. Data are presented as mean ± SEM. A two-way ANOVA using trial (oil exposure concentration) and treatment (control versus oil-exposed) as factors was used to determine significant differences in yolk sac depletion in developing mahi-mahi embryos. *Asterisks* indicate significant differences in measurements of yolk sac depletion area between control larvae and larvae exposed to different ΣPAHs for 24-hpf. Examples of yolk sac depletion area from control embryos raised in UV-sterilized seawater (left) and embryos exposed to a 4% HEWAF dilution for 24-hpf (right) (panel B).

could no longer keep up with energy demands required under these conditions (Figs. 3 and 4).

It is well established that activation of the aryl hydrocarbon receptor (AHR) pathway induces xenobiotic enzymes such as cytochrome P4501A (CYP1A) that are involved in the regulation and metabolism of aromatic hydrocarbons (Oziolor et al., 2014; Sørhus et al., 2016). In a comparative study between the spotted sea bass (*Lateolabrax maculates*) and the olive flounder (*Paralichthys olivaceus*), it was observed that flounder were less effective at inducing CYP1A and displayed more severe effects from oil exposure than the spotted sea bass, which displayed higher levels of both AhR and CYP mRNA expression (Jung et al., 2015). The regulation and induction of this detoxification mechanism comes at a cost not only energetically, but it has also been found to cause lethal cardiac deformities in both fish and mammals (Antkiewicz et al., 2006; Jönsson et al., 2007). We believe that the increased oxygen consumption observed in oil-exposed larvae is a response caused by the increase in metabolic demand necessary to fuel, among other things, these energetically expensive PAH metabolizing and detoxification processes. Indeed, CYP1A is among hundreds of genes changing expression in mahi-mahi embryos and larvae during oil exposure (Xu et al., 2016).

Synergistic impacts were predicted to occur when developing mahi-mahi embryos were exposed simultaneously to oil and elevated temperature due to faster development and higher basal metabolic rate at increased temperatures (Supplementary Fig. 5). These synergistic impacts and higher energy demands resulted in reduced hatch in oil-exposed embryos raised at 30 °C, whereas hatch rate in oil-exposed embryos raised at 26 °C did not differ from controls (personal observation). A possible explanation for the observed reduced hatch rate is that embryos raised at elevated temperatures did not have sufficient energetic reserves to fuel PAH detoxification mechanisms to prolong survival.

Increased nitrogenous waste excretion in treatment larvae supports our second hypothesis that increased metabolic demand in

oil-exposed developing mahi-mahi is fueled by protein catabolism (Fig. 5). Total nitrogenous waste excretion did not differ in embryos raised at 26 °C (Supplementary Fig. 3), which is to be expected as there were no changes in oxygen consumption at this developmental stage. A recent RNAseq study discovered upregulation of nitrogenous waste excretion transporters and enzymes, such as RHAG and RHBG, associated with pyrimidine metabolic pathways in oil-exposed developing mahi-mahi, reinforcing our hypothesis (Xu et al., 2016).

Although a trend for greater nitrogenous waste excretion in oil-exposed larvae compared to controls was apparent in all trials, oil-exposed larvae raised at 26 °C displayed significantly higher urea excretion, while larvae raised at 30 °C had significantly higher ammonia and total nitrogenous waste excretion (Fig. 5). A likely explanation of this finding was that larvae raised at higher temperatures were developing faster. The formation and build-up of ammonia is toxic and can be damaging to developing embryonic tissue (Wright and Fyhn, 2001). Many teleost fish have retained the genes for the urea cycle enzymes, even though they are inoperative in the liver of adults (Wright et al., 1995). Studies show that teleost fish such as the rainbow trout (*Oncorhynchus mykiss*) and the zebrafish (*Danio rerio*) accumulate both ammonia and urea as embryos before hatch (Steele et al., 2001; Braun et al., 2009), and it has been hypothesized that urea synthesis and excretion is a detoxification mechanism used to limit the build-up of ammonia in these developing embryos (Wright and Land, 1998). Therefore, many teleost fish are primarily ureotelic until hatch, and through development progressively increase ammonia excretion, becoming primarily or completely ammoniotelic at later-life stages (Wright et al., 1995). Mahi-mahi embryos are found to follow the same trend, switching from primarily ureotelic as embryos to primarily ammoniotelic as recently hatched larvae (Supplementary Fig. 3). Therefore, the discrepancy between data obtained at 26 and 30 ° is likely due to the fact that larvae raised at higher temperatures are developing faster and producing a greater proportion of their total nitrogenous waste as ammonia.

The relationship between exposure to tricyclic PAHs and disruption of cardiac development and function in fish has been demonstrated in multiple studies (Brette et al., 2014; Esbaugh et al., 2016; Incardona et al., 2004, 2009, 2014; Mager et al., 2014). The current study examined heart rate and pericardial and yolk sac edema as measures of cardiotoxicity to confirm the above exposure periods and concentrations produced effects similar to those reported previously in developing mahi-mahi and other species. Increased edema and reduced heart rate observed in this study suggest that larvae suffered from severe cardiac function impairment caused by oil exposure. Despite these findings, oil-exposed larvae exhibited significantly higher rates of oxygen consumption compared to controls (Fig. 1 and 2), illustrating that the circulatory system may not be the primary method of oxygen uptake and delivery in these yolk sac larvae. Control larvae raised at different temperatures had similar heart rates, despite significant differences in metabolic rate (Fig. 7), providing more support for the fact that larvae at this stage rely on alternative methods, i.e. cutaneous respiration and diffusion, for oxygen delivery. The apparent disconnect between decreased heart rate and increased metabolic rate may also indicate that mahi-mahi larvae increased stroke volume and cardiac output to compensate for increased energy demands. Future experiments are required to test this hypothesis.

Decreased hatch rate and faster metabolic rate were coupled with increased yolk sac depletion in mahi-mahi embryos raised at 30 °C, supporting synergistic effects of oil exposure and high temperature on metabolic demands (Fig. 8). The yolk sac is the sole provider of nutrition in developing fish until larvae are able to forage for food exogenously (Heming and Buddington, 1988). Mahi-mahi larvae open their mouth around 96-hpf and so up until this point must rely on the breakdown of their yolk sac for all nutritional and energetic needs. It seems likely that depletion of energy stores could reduce survival and fitness in early-staged mahi-mahi larvae and recent experiments documenting the toxicity of DWH crude oil on mahi-mahi embryos and larvae over the course of 96-h support this notion (Stieglitz et al., 2016). Future experiments documenting metabolism up to and past 96-hpf in mahi-mahi larvae are needed to fully understand the consequences of the documented increased energy demand associated with ELS oil exposure.

4.1. Caveats and limitations of the present study

Two to three trials were run at each exposure concentration at both temperatures tested in this study, to have the number of individuals required to run the above trials. Embryos were collected from one of two tanks containing multiple females and one male each. Additionally, trials were run over a 5-month period, producing slight variations in water conditions. Thus, genetic and environmental variability contributed to variability of the present data. Variability can be observed in differences in oxygen consumption, heart rate and pericardial and yolk sac edema in control mahi-mahi raised at the same temperatures. To reduce the risk of batch effects from confounding results, oil-exposed larvae were only compared to controls that came from the same cohort of embryos. Same batch embryos may still represent a mixed contribution from several females but were all spawned under identical conditions. Controls were only compared across trials when evaluating oxygen consumption, edema and heart rate between control mahi-mahi raised at different temperatures.

Nitrogen waste products in developing fish embryos are not constant and may be influenced by environment, developmental stage and amount of proteins and free amino acids (FAA) available. Variation in egg FAA content is small within a batch of embryos, but has been found to differ between batches and through the spawning period (Wright and Fyhn, 2001). Therefore, caution is recommended when comparing nitrogenous waste excretion between different trials.

4.2. Concluding remarks

The results of the current study provide evidence that oil exposure can be very damaging to developing mahi-mahi, producing a multitude of negative effects including cardiac impairment, increased energy demand and energy depletion and reduced hatch rate. The consequences these effects will have on individual mahi-mahi and mahi-mahi populations as a whole are largely unknown. Previous research has demonstrated that sublethal effects of oil exposure on ELS fish persist to later life stages. Both zebrafish and mahi-mahi exposed to oil for 48-h at ELS and raised in clean seawater displayed significant reductions in swim performance (U_{crit}) later in development (Hicken et al., 2011; Mager et al., 2014). Hicken et al., found that zebrafish embryos exhibiting edema generally failed to feed as larvae and survive metamorphosis (Hicken et al., 2011). Due to the severity of cardiac damage observed in the current study, oil-exposed larvae were not expected to persist to later stages, where a well functioning circulatory system becomes more critical for survival.

This study also demonstrates the importance of including multiple environmentally realistic stressors in toxicology tests. Most of the existing data on PAH effects on developing mahi-mahi are conducted at ambient, non-stressful temperatures, likely resulting in conservative estimations of damage.

Contributors

C.P. and M.G. designed the study. C.P performed the oxygen consumption, nitrogenous waste and energy depletion trials. C.P. and E.M.M. performed the heart rate and edema trials. J.D.S. and D.B. took care of the study animals and ensured healthy batches of embryos for every trial. C.P. wrote the manuscript. All authors provided insightful comments and feedback. M.G. secured funding.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.10.022.

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