Lack of biological effects of water accommodated fractions of chemically- and physically-dispersed oil on molecular, physiological, and behavioral traits of juvenile snapping turtles following embryonic exposure

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A B S T R A C T

Snapping turtle (Chelydra serpentina) eggs were exposed to two concentrations of chemically- or physically-dispersed water accommodated fractions of weathered Arabian light crude oil (Low = 0.5 and High = 10 g oil/L water). Solutions were passed through nest substrate to simulate alterations in composition during percolation to egg depth. Hatchlings were raised for 13 months during which numerous endpoints were measured. Prior to percolation, total PAH (“tPAH”; the sum of 52 PAHs measured) in physically-dispersed oil fractions were similar (High, 43; Low 67 mg/L). Following percolation, tPAH was also similar in physically-dispersed fractions (High, 14; Low 24 mg/L). Addition of dispersant increased tPAH prior to percolation in the High treatment (302 mg/L) relative to Low (13 mg/L), but percolation resulted in nearly equal concentrations in both treatments (High, 30; Low, 22 mg/L) due to physical trapping of dispersed oil by the nest substrate. In both chemically- and physically-dispersed fractions, percolation reduced low molecular weight (MW) compounds such that embryos were exposed to primarily mid- to high MW compounds. Total PAH in eggs differed 15-fold between the chemically-dispersed High and physically-dispersed High treatments (560 and 36 μg/kg, respectively), the former characterized by higher MW compounds than the latter. While eggs accumulated up to 560 μg/kg tPAH, we observed no effects on hatching success or hatching/juvenile traits (DNA integrity, survival, growth, metabolism, energy storage, or behavior), our results demonstrate that PAH profiles are altered during percolation, suggesting that experiments with subsurface organisms should be designed to account for compositional changes that occur as the solutions percolate through the substrate.

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1. Introduction

Oil spills near coastal habitats in temperate and tropical regions can contaminate areas used by aquatic turtles for nesting, especially during storm events or unusually high tides which may transport contaminants to the high beach nesting zone (Milton et al., 2003). Following an oil spill, wave and wind action entrains (disperses) or dissolves a portion of the floating slick into the water column (physical or natural dispersion). This fraction is chemically distinct from the overlying slick, typically being enriched in low molecular weight (MW) polycyclic aromatic hydrocarbons (PAH) relative to the parent oil. Arrival of the oil-contaminated parcel of water on beaches comprised of porous substrates provides potential for the oil slick or underlying water-entrained fraction to percolate through the substrate, exposing subsurface organisms to potentially toxic compounds. Furthermore, an oil-slicked beach may allow for continued percolation of water-entrained fractions after rain or further storm/high tide events.

The use of chemical dispersants to treat oil slicks is intended to increase entrainment of droplets and dissolution of some compounds from the slick into the water column. This treated fraction containing a greater proportion of mid to high MW compounds and oil droplets differs compared to that produced when dispersants are not added. Dispersants are typically employed to treat oil slicks in marine open water systems in which constituents of the treated oil slick become highly diluted prior to entering shallow or coastal systems. However, consideration of the use of dispersants in near shore habitats raises concerns regarding effects on shallow benthic habitats and beaches (NRC, 2005). The addition of a dispersant creates a sub-surface plume of the dispersant itself, as well as PAHs in the dissolved, colloidal, or particulate phases (fine oil droplets) which rapidly migrates to a greater depth than the plume of PAHs produced from natural dispersion (NRC, 2005). Thus following addition of dispersant to a slick, organisms inhabiting benthic habitats may be at risk of exposure...
at depths at which they would not otherwise be exposed. Furthermore, for subsurface, beach-dwelling organisms, the action of dispersants to produce fine droplets of oil would be expected to provide a possible route for delivery of the parent oil itself to greater depth than may occur with non-dispersed oil. As a result, percolation of dispersant-treated oil through beach substrates may expose buried organisms (such as turtle eggs) to a greater total concentration of oil-derived compounds than the naturally-dispersed oil plume or the residual slick, as well as exposing them to a suite of compounds different from those present in a naturally-dispersed fraction (including the dispersant itself). Relative to the parent oil or physically-dispersed fraction, chemically-dispersed oil may thus induce different types or magnitudes of effects on subsurface organisms than those induced by exposure to constituents of the naturally-dispersed plume or the residual slick.

Sea turtles typically nest in sandy areas of high beaches, and coastal oil spills may expose nests to PAHs or PAHs/dispersant-derived compounds that percolate through the substrate. Shipping accidents and associated oil spills frequently occur during times of storms and heavy surf (NOAA, 2003), allowing transport of oil to the high beach zone, where turtle nests are typically constructed. While the initial contamination event may be somewhat brief (e.g., the spill itself will disperse relatively rapidly naturally or through enhancement by dispersants), residual oil-derived compounds entrained in the matrix of the nest substrates may chronically expose turtle eggs to potentially toxic compounds.

Saturation of turtle nests with oil is known to induce mortality in embryos. For example, following a spill that released 336,000 gal of fuel oil in Tampa Bay, Florida in 1993, producing a slick covering 23 km of sandy beach, loggerhead sea turtle nests heavily saturated with the oil produced survival rates of ~5%, compared to a normal rate of 50 to 90% (Yender and Mearns, 2003). Whether effects of direct exposure to oil reflect toxic effects on the embryos or simply suffocation due to reduced gas exchange across the egg shells, however, remains unknown (e.g., Phillott and Parmenter, 2001; Yender and Mearns, 2003).

Despite observations from such natural experiments, controlled dose–response studies of the severity and possible residual effects of compounds present in oil and dispersant-treated oil fractions that percolate to the eggs have not been conducted. The lack of experimental data from studies in which exposures were controlled and conducted under conditions similar to those in natural sites precludes assessing how sensitive turtles may be to oil-derived contaminants, as well as how effects would be expressed physiologically. This information gap critically constrains spill responders and regulators in determining the proper remediation strategy (e.g., whether to use dispersants in near shore systems) to employ following oil spills to best protect turtle nests.

The threatened or endangered status of sea turtles worldwide precludes large scale toxicity studies on them for both ecological and ethical reasons. Use of surrogate species that are abundant and share traits with sea turtles provides an alternative to manipulating at-risk species while serving as models from which experimental results can be applied to other species. We conducted a study in which common snapping turtles (Chelydra serpentina) were used as surrogates for sea turtles and many sea turtles are relatively slight (Packard et al., 1982). In contrast to many other turtle species that produce rigid shelled eggs, those structural differences between eggs of snapping turtles and many sea turtles are relatively slight (Packard et al., 1982). However, as eggs shells of snapping turtles possess more defined pores than those of sea turtles (Packard et al., 1982), eggs of the former may be somewhat more prone to accumulation of fluid-borne or volatile contaminants than the latter. Thus it is possible that the snapping turtle may be more likely than sea turtles to respond to exposure to oil-derived compounds in the nest, providing conservative estimates of effects on sea turtles. Furthermore, snapping turtles, like all sea turtles and most other turtles, possess temperature dependent sex determination (TSD) rather than genetic sex determination (GSD), and thus the potential for exposure to contaminants during the embryonic period to modify sexual development through altering hormonal dynamics. As PAHs are known to exhibit estrogenic activities (e.g. Garcia-Reyero et al., 2005), the existence of TSD in snapping turtles suggests that they may be useful models to study the effects of altered endocrinological processes resulting from PAH exposure on sexual development of reptiles.

2. Methods

2.1. Study species

The freshwater/brackish water common snapping turtle possesses traits that make it a useful surrogate species for sea turtles. Snapping turtles are locally abundant in North America and produce large numbers of flexible shelled eggs of similar structure to most sea turtles, although the latter tend to have a slightly more loosely organized calcareous matrix and less well defined pores (Packard et al., 1982). In contrast to many other turtle species that produce rigid shelled eggs, those structural differences between eggs of snapping turtles and many sea turtles are relatively slight (Packard et al., 1982). However, as eggs shells of snapping turtles possess more defined pores than those of sea turtles (Packard et al., 1982), eggs of the former may be somewhat more prone to accumulation of fluid-borne or volatile contaminants than the latter. Thus it is possible that the snapping turtle may be more likely than sea turtles to respond to exposure to oil-derived compounds in the nest, providing conservative estimates of effects on sea turtles. Furthermore, snapping turtles, like all sea turtles and most other turtles, possess temperature dependent sex determination (TSD) rather than genetic sex determination (GSD), and thus the potential for exposure to contaminants during the embryonic period to modify sexual development through altering hormonal dynamics. As PAHs are known to exhibit estrogenic activities (e.g. Garcia-Reyero et al., 2005), the existence of TSD in snapping turtles suggests that they may be useful models to study the effects of altered endocrinological processes resulting from PAH exposure on sexual development of reptiles.

2.2. Study design

The protocol that we employed was designed to identify the effects of embryonic exposure to oil either having been chemically dispersed through addition of a dispersant or having not had dispersant added on snapping turtles incubated in artificial nests in the laboratory. The oil used was an Arabian light crude oil (generously provided by Dr. Al Venosa, USEPA) which we weathered by a 20% loss evaporative by volume to simulate approximately 24 h of weathering. Treatments consisted of four replicates each of two concentrations of chemically dispersed or physically-dispersed oil, a filtered (0.2 μm) water-only control, and control water containing only the dispersant (Corexit 9500™). An additive (J. Clark, pers. com., George-Ares et al., 2001) was added in order that the dispersant would be effective in freshwater, as snapping turtle embryos are unlikely to be tolerant of highly saline water. In preparing the solutions, we followed the standardized CROSERF protocol (Singer et al., 2000) to produce the fraction of oil that would naturally enter the water column (water accommodated fraction — “WAF”) and that which would result from addition of dispersant (chemically enhanced water accommodated fraction — “CEWAF”). Nominal concentrations of WAF and CEWAF were 0.5 (WAF and CEWAF “Low”) and 10 g oil/L water (WAF and CEWAF “High”). Mixing and ratios of dispersant to oil followed the CROSERF standardized test protocol (Singer et al., 2000). Briefly, weathered Arabian light crude oil and where appropriate a 1:20 (dispersant:oil) volume of Corexit 9500 (with additive), were added to glass (10L) aspirator bottles and the containers sealed. Mixing (approx. 25% vortex depth using stir bars) was carried out for 24 h in the dark at the exposure experiment temperature (25 ± 1°C). After 24 h the mixing was stopped and the oil/water/dispersant mixtures were allowed to separate for 6 h. After 6 h the exposure test solutions were carefully drained from the bottom of the vessels, leaving the ‘slick’ behind. These stock solutions were sub-sampled for PAH chemical analyses. The exposure solutions were then prepared by percolating the initial stock solutions through 18 cm of sand/gravel (see below). Sub-
samples of these exposure solutions following percolation were also analyzed for PAHs.

2.3. Exposure protocol

We collected eggs in May, 2006 from a commercial turtle farm in Louisiana and transported them to our laboratory in MD. After a two week holding period, eggs were placed in shallow nests (3 cm) deep, a technique that has provided good hatching success in the past (Rowe, pers. obs.). Eggs from 12 clutches were distributed evenly among all nests such that eggs from each clutch were represented in all treatments. Half of the nests (2 per treatment) contained 2 PAH binding substrates (Amberlite XAD™ resin) in paper tea-bag material to assess PAH congener patterns in solutions when present in the vicinity of the buried eggs. Two additional eggs were placed in 2 nests per treatment (with the exception of WAF Low and CEWAF Low due to analytical cost constraints) for analysis of accumulation of PAHs by embryos over the first six days following addition of solutions, when they had presumably been exposed to the highest concentrations of PAHs. Dosing solutions (1 L per nest) were first passed through an 18 cm long column containing nest substrate (3 parts rinsed medium sand to 1 part pea gravel) prior to addition of 1 L of the solution to each nest. This protocol was employed so that the solutions delivered to the eggs would reflect alterations to concentrations or composition that would occur as the solutions percolate to eggs in a nest. Samples from stock solutions prior to and following percolation through the sand column were collected from two replicate nests per treatment and analyzed as described below.

Following dosing nests were housed in a dark, 25 °C constant-temperature room until hatching. The incubation temperature was chosen as it typically produces predominately male hatchlings (Yntema, 1976), thus providing for assessment of potential sex reversal following embryonic exposure to PAHs. Six days after dosing, the overlying substrate was removed and replaced with moist, autoclaved sphagnum moss, and one XAD substrate and the 2 additional eggs were removed for chemical analyses. The remaining XAD substrates were removed at the onset of hatching. The sphagnum moss was misted with water as necessary to maintain moisture throughout the remainder of the experiment.

2.4. Biological endpoints

2.4.1. Hatching success/embryonic mortality

At 2 to 3 day intervals, the overlying moss was removed from each nest and eggs inspected for viability (normal coloration and hydration) and indications of imminent hatching (sloughing of outer shell layers). Non-viable eggs were noted and discarded. Moss was replaced at approximately 2 week intervals. As eggs pipped (shell began to split) they were removed to individual 0.5 L polyethylene containers containing moist sphagnum until hatching was complete.

2.4.2. Post-hatching endpoints

2.4.2.1. COMET assay (genotoxicity). As PAHs are known to illicit DNA strand breakage (Mitchelmore et al., 1998; Taban et al., 2004), we assessed DNA damage on a subset of hatchlings. One hatching from each of 3 nests per treatment was sacrificed one week after hatching for assessment of DNA damage in a portion of liver tissue. DNA damage was assessed by the comet assay (see Mitchelmore and Chipman, 1998; Livingstone et al., 2000). Briefly, livers were washed with ice cold HEPES HBSS solution (pH 7.6). Tissues were minced with a Teflon-coated razor blade and passed through a 70μm filter. 10μL of the filtrate containing cells was mixed with 100μL of 0.75% low-melting point agarose (in HEPES/HBSS buffer) and placed on a pre-coated slide of dried 1% NMA agarose. Triplicate slides from each turtle liver were prepared. The rest of the solution was used to assess cell viability using the trypan blue exclusion method. After solidification slides were coated with a further 100μL of 0.75% LMPA, allowed to solidify and then placed in ice-cold lysis solution for 1 h. Following lysing, slides were rinsed and placed in an electrophoresis chamber containing unwinding buffer for 10 min. Slides were then electrophoresed for 10 min at 25 V, 300 mA. Slides were rinsed three times with neutralization buffer, stained with 20μg/μL Ethidium bromide and viewed under a fluorescent scope and

Table 1
Specific congeners, molecular weights (MW) and log Kow of PAHs quantified.

<table>
<thead>
<tr>
<th>Congener</th>
<th>MW</th>
<th>log Kow</th>
<th>Congener</th>
<th>MW</th>
<th>log Kow</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Naphthalene</td>
<td>128.00</td>
<td>3.30</td>
<td>27. 1-Methylphenanthrene</td>
<td>192.00</td>
<td>5.14</td>
</tr>
<tr>
<td>2. 2-Methylphenanthrene</td>
<td>142.00</td>
<td>3.86</td>
<td>28. 9-Methylanthracene</td>
<td>192.00</td>
<td>5.07</td>
</tr>
<tr>
<td>3. Azulene</td>
<td>128.00</td>
<td>3.20</td>
<td>29. 9,10-Dimethylanthracene</td>
<td>206.00</td>
<td>5.25</td>
</tr>
<tr>
<td>4. 1-Methylphenanthrene</td>
<td>142.00</td>
<td>3.86</td>
<td>30. Fluoranthene</td>
<td>202.00</td>
<td>5.22</td>
</tr>
<tr>
<td>5. Biphenyl</td>
<td>154.00</td>
<td>3.97</td>
<td>31. Pyrene</td>
<td>202.00</td>
<td>5.16</td>
</tr>
<tr>
<td>6. 2,7-Dimethylphenanthrene</td>
<td>156.23</td>
<td>4.31</td>
<td>32. 3,6-Dimethylphenanthrene</td>
<td>206.00</td>
<td>5.44</td>
</tr>
<tr>
<td>7. 1,3-Dimethylphenanthrene</td>
<td>156.23</td>
<td>4.42</td>
<td>33. Benzo[a]fluorene</td>
<td>216.00</td>
<td>5.32</td>
</tr>
<tr>
<td>8. 1,6-Dimethylphenanthrene</td>
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<td>4.44</td>
<td>34. Retene</td>
<td>234.34</td>
<td>6.35</td>
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<td>9. 1,4-Dimethylphenanthrene</td>
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<td>35. Benzo[b]fluorene</td>
<td>216.00</td>
<td>5.77</td>
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<td>10. 1,5-Dimethylphenanthrene</td>
<td>155.23</td>
<td>4.26</td>
<td>36. Cyclopenta[c,d]pyrene</td>
<td>222.29</td>
<td>5.70</td>
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<tr>
<td>11. Acenaphthylene</td>
<td>152.00</td>
<td>3.90</td>
<td>37. Benzo[a]anthracene</td>
<td>228.00</td>
<td>5.91</td>
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<tr>
<td>12. 1,2-Dimethylphenanthrene</td>
<td>156.23</td>
<td>4.31</td>
<td>38. Chrysene + Triphenylene</td>
<td>228.00</td>
<td>5.79</td>
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<tr>
<td>13. 1,8-Dimethylphenanthrene</td>
<td>156.23</td>
<td>4.26</td>
<td>39. Naphthacene</td>
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<td>5.84</td>
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<td>154.00</td>
<td>3.97</td>
<td>40. 4-Methylchrysene</td>
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<td>6.07</td>
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<td>15. 2,3,5-Trimethylphenanthrene</td>
<td>170.25</td>
<td>4.81</td>
<td>41. Benzo[b]fluoranthene</td>
<td>252.00</td>
<td>6.44</td>
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<td>16. Fluorene</td>
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<td>4.18</td>
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<td>252.00</td>
<td>6.44</td>
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<td>17. 1-Methylfluorene</td>
<td>180.00</td>
<td>4.97</td>
<td>43. Dimethylbenzo[a]anthracene</td>
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<td>18. Dibenzo[a]fluorene</td>
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<td>4.38</td>
<td>44. Benzo[e]pyrene</td>
<td>252.00</td>
<td>5.98</td>
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<td>19. Phenanthrene</td>
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<td>4.57</td>
<td>45. Benzo[a]pyrene</td>
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<td>6.44</td>
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<tr>
<td>20. Anthracene</td>
<td>178.00</td>
<td>4.54</td>
<td>46. Perylene</td>
<td>252.00</td>
<td>6.44</td>
</tr>
<tr>
<td>21. 2-Methylbenzothiophene</td>
<td>192.00</td>
<td>4.71</td>
<td>47. 3-Methylcholanthrene</td>
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<td>7.11</td>
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<td>22. 4-Methylbenzothiophene</td>
<td>198.28</td>
<td>4.71</td>
<td>48. Dibenzo[a,h +ac]anthracene</td>
<td>276.00</td>
<td>7.04</td>
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<td>23. 2-Methylphenanthrene</td>
<td>192.00</td>
<td>4.86</td>
<td>49. Benzo[a]pyrene</td>
<td>276.00</td>
<td>7.10</td>
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<td>24. 2-Methylanthracene</td>
<td>192.00</td>
<td>5.00</td>
<td>50. Benzo[k]pyrene</td>
<td>276.00</td>
<td>7.04</td>
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<tr>
<td>25. 4,5-Methylenehexanthrene</td>
<td>190.00</td>
<td>4.60</td>
<td>51. Anthracene</td>
<td>276.00</td>
<td>7.04</td>
</tr>
<tr>
<td>26. 1-Methylanthracene</td>
<td>192.00</td>
<td>4.89</td>
<td>52. Corenene</td>
<td>300.00</td>
<td>7.64</td>
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</table>
quantified using KOMET 5.0 image analysis. Results are reported as tail % DNA, tail length and tail moment.

2.4.2.2. Survival and growth. The number of hatchlings produced in each nest was counted to determine embryonic survival. Following hatching and resorption of residual yolk, animals were held individually in a 20–24 °C laboratory in 1 L polyethylene containers filled to approximately 5 cm depth with well water for the duration of the study (13 months post-hatching). Survival was monitored daily and growth was measured monthly as carapace length (CL; straight line distance from the anterior- to posterior-most carapacial scutes) and wet weight (WW). As growth trajectories did not differ, only final sizes are presented and discussed here. Hatchlings were fed commercial turtle food (Fluker Laboratories, Baton Rouge, LA) ad libitum every 2 days. Water in the holding containers was changed weekly.

At the end of the experiment, juveniles were sacrificed by inhalation of an anesthetic (isofluorane) followed by decapitation. Animals were dissected for sex identification based upon gonadal morphology (Yntema, 1976), and the livers were removed for calculation of hepatosomatic indices (HSI; ratio of liver WW to carcass WW).

2.4.2.3. Bioenergetic endpoints. We quantified bioenergetic properties of hatchlings (metabolism, energy storage) due to the overall narcotic effects induced by some PAHs (e.g. McCarty et al., 1992)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stock, prior to percolation (mg/L)</th>
<th>Stock, following percolation (mg/L)</th>
<th>Egg contents (μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>4.8</td>
</tr>
<tr>
<td>Dispersant</td>
<td>1</td>
<td>DN M</td>
<td>5.9</td>
</tr>
<tr>
<td>WAF Low</td>
<td>67</td>
<td>24</td>
<td>DN M</td>
</tr>
<tr>
<td>WAF High</td>
<td>43</td>
<td>14</td>
<td>36.4</td>
</tr>
<tr>
<td>CEWAF Low</td>
<td>13</td>
<td>22</td>
<td>DN M</td>
</tr>
<tr>
<td>CEWAF High</td>
<td>302</td>
<td>30</td>
<td>563</td>
</tr>
</tbody>
</table>

Stock concentrations in mg/L, egg concentrations in μg/kg. DN M = did not measure.

Table 2

Fig. 1. Congener patterns and total PAH (mg/L) in WAF and CEWAF solutions, prior to and after percolation through the substrate. A. WAF Low prior; B. WAF High prior; C. WAF Low after; D. WAF High after; E. CEWAF Low prior; F. CEWAF High prior; G. CEWAF Low after; H. CEWAF High after. Note the differences in scales. Congener numbers on the horizontal axis correspond to designations in Table 1.
which can potentially reduce metabolism, growth, and storage of energy as lipids. Metabolic rates of a subset of four to five hatchlings from each replicate nest were measured as O₂ consumption at rest in post-absorptive individuals by microrespirometry (MicroOxy-max™, Columbus Instruments, Columbus, OH). We chose individuals from clutches that were represented in all nests in order to reduce variability due to maternal effects on bioenergetics (Steyermark and Spotila, 2001). Hatchlings were acclimatized to 20 °C for 48 h prior to measurement of metabolic rates, during which time they were not fed. Metabolic rates were measured on the same individuals in each of 3 trials (6, 10, and 11 months post hatching). During measurement of oxygen consumption, hatchlings were held individually in 500 mL jars containing a damp, unbleached paper towel in an unlit, 20 °C incubator. Eight to 10 measurements were made at ~3 h intervals for 24 to 30 h, following which individuals were weighed and returned to the holding laboratory.

Energy storage (total body lipid content) was measured non-destructively on the same subset of hatchlings used for measurement of oxygen consumption using total body electrical conductivity (TOBEC Model SA-2, EM SCAN, Springfield, IL; see Walsberg, 1988; Kintner and Johnson, 1998) at 6, 9, and 11 months post hatching. Using carcasses from a prior study with the same species, we derived the following algorithm as a best fit relationship between electrical conductivity and body composition:

\[
\% \text{ wet weight comprised of lipid} = -9.975 + 0.216E - 0.001E^2,
\]

where \( E \) = electrical conductivity parameter produced by the instrument (\( R^2 = 0.974, P < 0.001 \)). These values were then corrected for body water content for comparison with other studies in which body composition are typically reported based upon percentage of lipid per unit dry body weight. Note that values that we calculated using this technique were comparable to those reported for hatchling and juvenile turtles for which lipids were quantified using destructive methods (Rowe et al., 1995; Nagle et al., 1998).

2.4.2.4. Behavioral assays. Potential irregularities in neurological development were measured using two behavioral assays on hatchlings. On two occasions (8 and 10 months post hatching) we assessed the response of juveniles to a visual stimulus using a technique adapted from Winkelman (1996), in which a simulated avian predator was used to cast a shadow on or near the test animal. Six individuals per treatment, consisting of 1 or 2 per replicate nest were randomly selected and each was placed individually in a 38 L aquarium filled to

![Fig. 1 (continued).](image-url)
4 cm water depth and lighted by a unidirectional light source overhead. A bird-shaped silhouette was passed through the light path in a standardized fashion from behind a blind to cast a shadow on the turtle. Three assays were run per individual on each occasion. Response behaviors were scored based upon relative activity level: no response, head twitch, full body twitch, or active swimming. Each activity was scored as either having occurred (assigned a score of 0) or not occurred (1) for each assay on each individual and the mean of the three scores per response calculated for each behavior. On one occasion we also assessed the righting response of individuals (the time required for the individual to return itself from a ventrally-upright to normal position; e.g. Steyermark and Spotila, 2001). We placed individuals on their back on a packed sand substrate and monitored times to righting. Measurements were made under indirect light, and observations were made from behind a blind.

2.5. Chemical analysis

Initial stock solutions: stock solutions before and following percolation through the sand column, XAD substrates, and egg contents were analyzed for 52 PAHs (Table 1). Methodology largely followed protocols described by Ko and Baker (1995) for XAD and egg samples, and by Reddy and Quinn (1999) for liquid samples. XAD and egg samples were extracted for 24 h in a Soxhlet apparatus with a 1:1 (v:v) acetone; hexane solvent following grinding with pre-extracted Na₂SO₄. Acetone was subsequently removed by liquid–liquid partitioning into deionized water and the remaining extract concentrated using rotary evaporation. Stock solutions were extracted with 100 mL dichloromethane and twice with 100 mL hexane.

Extracts were concentrated in hexane and eluted through a baked (550 °C, 4 h minimum) silica gel column consisting of approximately 3 g of silica gel packed into a 10 mL pipette and topped with sodium sulfate. Columns were pre-eluted with 20 mL 80:20 hexane:dichloromethane and eluted with 20 mL of the same mixture. Ultra high purity nitrogen was used to push samples through the column, which removed polar compounds prior to purified extracts being analyzed by GCMS. PAHs were identified and quantified using a capillary gas chromatograph (Hewlett Packard 5890) and a mass spectrometer (Hewlett Packard 5970A) operated in selected ion monitoring mode with helium as the carrier gas. PAHs were identified by retention time relative to that of mixed standards (Supelco Separation Technologies, Bellefonte, PA). Identification was confirmed by the abundance of a secondary mass fragment relative to the molecular ion.
Surrogate PAH standards (perdeuterated PAHs d₈-naphthalene, d₁₀-fluorene, d₁₀-fluoranthene, and d₁₂-perylene) were added to each sample prior to extraction to quantify overall method performance. Internal standards (d₁₀-acenaphthalene, d₁₀-phenanthrene, d₁₂-benzo[a]anthracene, d₁₂-benzo[a]pyrene, d₁₂-benzo[g,h,i]perylene) were added to samples and calibration standards prior to analysis and the instrument was calibrated by comparing the response of each analyte to that of the respective internal standard. Surrogate recoveries ranged from 64% (naphthalene) to 109% (fluorene) and did not vary systematically across treatments.

2.6. Statistical analyses

Survival, growth, righting response, and DNA damage were analyzed by single factor analysis of variance (ANOVA) followed by Tukey's pairwise comparisons. For responses that may vary allometrically with animal size (metabolic rate, body composition) we employed analysis of covariance (ANCOVA) using wet weight as the covariate. To assess predator avoidance, the average number of each response over the three replicate trials on each individual were calculated and treatments compared using multiway analysis of variance (MANOVA). Statistical significance was judged based upon a Type I error rate of α = 0.05. Prior to statistical analyses, data were tested to verify that assumptions of the models were met and transformed if necessary. Data for PAH concentrations in solutions, eggs, and XAD substrates were not analyzed statistically because, due to costs associated with chemical analyses, only 2 samples per treatment were measured.

3. Results

Total PAH concentrations in stock solutions prior to and following passage through the sand column and the patterns of specific congener constituents are presented in Table 2 and Fig. 1. Concentrations of tPAH in the Low and High WAF stocks were similar between

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Fig. 1 (continued).

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3. Results

Total PAH concentrations in stock solutions prior to and following passage through the sand column and the patterns of specific congener constituents are presented in Table 2 and Fig. 1. Concentrations of tPAH in the Low and High WAF stocks were similar between
doses prior to percolation (67 and 43 mg/L). Total PAH in Low and High WAF stocks were also similar after percolation (24 and 14 mg/L). In both of these Low and High WAF stocks, tPAHs were reduced (2.8 and 3.1-fold respectively) by percolation. This reduction in tPAHs following percolation of the WAF stocks was mainly due to a reduction in concentrations of low molecular weight (MW) PAHs. CEWAF Low and High stocks differed considerably prior to percolation between the two doses used (Low = 13 mg/L; High = 302 mg/L; not analyzed statistically) and both doses were enriched in higher MW compounds relative to WAF stocks prepared with the same amount of oil.

Percolation substantially reduced (over 10-fold) tPAH concentration in CEWAF High (prior to percolation = 302 mg/L; following percolation = 30 mg/L). Following percolation through the sand substrate, a greater proportion of PAHs ranging from approximately log $K_{ow}$ of 4.5 to 5.5 (i.e. phenanthrene through pyrene; Table 1) were retained in the sand treated with CEWAF than with WAF (Fig. 1). Total PAH concentrations were similar between CEWAF and WAF after percolation through the sand (range 14–30 mg/L for the four treatments; Fig. 1), although in CEWAF there was a greater proportional removal of the “mid $K_{ow}$” compounds remaining relative to WAF.

XAD substrates were used only to compare congener patterns rather than absolute levels of exposure among treatments due to potential differences in absorption of solutions among nests. Analysis of PAH in XAD substrates buried near the eggs illustrated that over the course of the study, the PAH profiles in CEWAF treatments shifted somewhat toward mid-molecular weight congeners, together with a depletion of low molecular weight compounds (e.g. anthracene and below; Fig. 2). However, in both the CEWAF and WAF treatments, the PAH congener patterns at egg-depth were still strongly skewed toward low to mid-molecular weight compounds (Fig. 2). As several XAD bags disintegrated over time, we have no profiles of PAH congeners in XAD from CEWAF High at the beginning of the study or at

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**Fig. 3.** Congener patterns and total PAH (μg/kg) in eggs six days following addition of solutions to nests. A. WAF High; B. CEWAF High. Note the differences in scales. Congener numbers on the horizontal axis correspond to designations in Table 1.
the time of hatching, nor do we have profiles for dispersant-only treatment at the time of hatching.

Concentrations of tPAH in eggs removed six days following dosing revealed that eggs treated with CEWAF High accumulated much greater concentrations than did those from the WAF High, Dispersant, or Controls (Table 2, Fig. 3). Concentrations of tPAH in eggs from WAF High were only ~6.5% of those in CEWAF High.

Hatching success was high in all treatments. CEWAF High exposure resulted in the lowest hatching success (60%), yet it did not differ statistically from other treatments (P = 0.05; Table 3). The duration of the embryonic period was very consistent and did not differ among treatments, varying only by an average of one day among treatments (Table 3). Neither WW nor CL of hatchlings differed among treatments. Despite accumulation of PAHs in eggs (CEWAF High in particular), there was no evidence of elevated DNA damage in hatchlings from any treatment (Table 3). Sizes of juveniles at the end of the study did not differ among treatments (Table 3), nor was there evidence of behavioral anomalies in juveniles from any treatment.

There were no differences in metabolic rate among treatments during any trial. In the initial assay of body lipid content (7 months post hatch), lipid content of hatchlings from the WAF Low treatment was greater concentrations than did those from the WAF High, Dispersant, or Controls (Table 2, Fig. 3). Concentrations of total body lipid content among treatments in the assays conducted at 9 and 11 months post hatch, despite what appeared to be a considerable reduction in lipid content in CEWAF High relative to other treatments.

Mean survival of hatchlings through the end of the study ranged from 54 to 76% among treatments, being lowest on average in CEWAF High relative to other treatments, although the differences were not statistically significant (Table 3). There were no differences in HSI or sex ratios of hatchlings among treatments (Table 3). We observed few gross abnormalities in any treatment.

4. Discussion

Our study demonstrates that, relative to corresponding WAF treatments, CEWAF treatments delivered considerably higher concentrations of total PAH to nesting substrates and to egg contents. As would be expected, treatment with the dispersant (CEWAF) resulted in enrichment in somewhat higher molecular weight congener concentrations in dosing solutions relative to non-dispersed, WAF treatments. Passage of CEWAF solutions through the sand column substantially reduced the total PAH concentrations delivered to the eggs (particularly in CEWAF High). Retention of PAHs from CEWAF in the sand likely reflected the presence of colloids of high MW compounds produced by the dispersant which likely adhered to particles in the substrate. On the other hand, total PAH concentrations in WAF solutions were less affected (i.e. 1.4 versus 10-fold in WAF and CEWAF High respectively) by percolation, having originally had much lower concentrations of PAH than the CEWAF High solutions and being characterized by lower compounds of lower MW and log KOW which would be more likely to be transported with water through the substrate.

Analyses of egg PAH concentrations revealed that those exposed to the highest CEWAF solutions accumulated much higher concentrations than those in WAF, although statistical analyses were not conducted (N = 2 composite samples per treatment). Eggs from the WAF High treatment accumulated primarily mid-molecular weight PAHs (phenanthrene through pyrene), likely reflecting selective removal of low molecular weight congeners by percolation through the substrate. CEWAF High eggs accumulated low through mid-molecular weight PAHs and the total PAH concentrations delivered to the substrate surrounding the eggs and thus being available for accumulation.

Despite what appeared to be trends suggesting that juveniles from CEWAF High treatments experienced lower lipid content late in the study and perhaps reduced survival, these effects were not statistically significant. Despite accumulation of low to mid-molecular weight congeners by eggs (particularly when exposed to CEWAF) which have been shown to induce toxic responses such as genetic damage in other organisms (Incardona et al., 2005), we observed a general lack of significant responses in embryos or hatchlings. However, PAHs vary substantially in their toxicity among congeners and taxa (e.g. Eisler, 1987). Birds for example (mallard ducks) exposed embryonically to PAHs were most substantially affected by the high molecular weight congeners dimethylbenz(a)-anthracene (log KOW = 5.8), which induced embryonic toxicity at a concentration of only approximately 36 μg/kg ww (Hoffman and Gay, 1981). This compound was present
at very low concentrations (below analytical detection limits) in the stock solutions and eggs in our study. As well, PAH profiles of eggs in our studies were dominated by congeners that have been shown to have little or no effects on the aryl hydrocarbon receptor in fish ("Ahr," an indicator of onset of toxicological response), which tends to respond to higher molecular weight compounds (Barren et al., 2004). Additionally, Matson et al. (2005) reported correlations between environmental concentrations of low molecular weight 3-ring congeners (fluorene through anthracene) and DNA damage in adult turtles in Azerbaijan. These compounds were present in eggs in our study, yet we observed no effects on DNA strand integrity in hatchlings. The sites in which Matson et al. (2005) observed prevalent DNA damage had total low molecular weight 3-ring PAH concentrations of 165 μg/kg in sediment (tissue concentrations not reported), whereas the highest total concentration of these congeners in egg contents in our study was 50 μg/kg (in CEWAF High; Fig. 3D).

While PAH exposures did not induce toxicological effects in our studies, turtles and other reptiles have been shown to be sensitive to some organic and inorganic contaminants under natural exposure regimes. Exposure of turtle eggs to PCBs, organic pesticides, and heavy metals via maternal transfer have been shown to affect sexual development, metabolic efficiency, hatchling success, morphological development, and long term survival (Bishop et al., 1998; Nagle et al., 2001; de Solla et al., 2002; Rowe, 2008). With respect to PAH exposure specifically, a recent study of turtle eggs and hatchlings collected from a contaminated site suggested a relationship between total PAH in fat and hatching deformities (Bell et al., 2006). Total PAH in fat was reported as 2.3 to 4.5 mg/L, an order of magnitude higher than the highest total PAH concentration that we measured in whole egg samples (0.56 mg/L in CEWAF High). As many as 50% of embryos and hatchlings collected from the contaminated site displayed deformities, suggesting that exposure to such high concentrations of PAH during development may have been responsible. However numerous other organic and inorganic compounds were also present at the site (but not measured in turtles), thus confounding interpretation of direct relationship between PAH and deformities.

The highest exposure concentrations in this study were likely much higher than the ones that would be expected to be present on a beach following a spill (NRC, 2003, 2005), yet none of the numerous endpoints induced responses that would be expected to compromise health or fitness. Under the specific conditions in which we conducted our experiments, and based upon the suite of endpoints that we quantified, it appears that the developing turtles were quite tolerant of exposure to compounds derived from WAF and CEWAF, as well as the dispersant itself. However, as there have been no dose–response studies correlating embryonic effects in turtles with PAH exposure, we cannot interpret the accumulated concentrations of total PAH or specific congeners relative to known thresholds of effects.

Whether the snapping turtle was an adequate model for estimating effects on sea turtles cannot be explicitly determined. As the structure of the egg shell of snapping turtles is characterized by abundant, better defined pores relative to sea turtles (Packard et al., 1982), we predicted that accumulation by snapping turtle eggs may exceed that of sea turtle eggs, providing a conservative estimate of exposure of the latter. Thus, assuming that response correlates with accumulation, sea turtle eggs would be expected to be less affected than our surrogate species when exposed to the same solutions. However, we cannot generally conclude that turtle embryos are universally tolerant of exposure to oil derived hydrocarbons, as we examined a specific scenario in which eggs were exposed only to WAF or CEWAF from one type of crude oil, and we did not examine the residual slick that may also be present following a spill which may present the additional stress of reduced gas transport and the possibility of suffocation (Philott and Parmenter, 2001). Furthermore, we did not vary the developmental stage at which exposure first occurs nor the potential interactive effects of natural stressors (temperature and moisture fluctuations, for example). On the other hand, that incorporated percolation of exposure solutions through a substrate to approximate exposures as they would occur in natural nests presumably provided realistic exposure situations with respect to the specific compounds that would be expected to come into contact with eggs in natural settings. Thus, while the design of our study incorporated exposure regimes that approximated those that would occur in natural systems in the case in which a slick or emulsion does not cover the nests which may result in repeated percolation events during rain events, we cannot provide definitive evidence that sea turtle eggs are unaffected by exposure to WAF and CEWAF in natural habitats.

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