

Loggerhead sea turtle (*Caretta caretta*) egg yolk concentrations of persistent organic pollutants and lipid increase during the last stage of embryonic development

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Received 13 December 2005; received in revised form 14 February 2006; accepted 21 February 2006

Available online 6 March 2006

Abstract

Data are scarce describing the concentrations of polychlorinated biphenyls (PCBs) and organochlorine pesticides in sea turtle eggs. The purpose of this study was to establish appropriate sample collection methodology to monitor these contaminants in sea turtle eggs. Contaminant concentrations were measured in yolk samples from eggs that failed to hatch from three loggerhead sea turtle (*Caretta caretta*) nests collected in southern Florida to determine if concentrations change through embryonic development. One to three egg yolk samples per nest were analyzed from early, middle, and late developmental stages ($n=22$ eggs total). PCB and pesticide concentrations were determined by gas chromatography with electron capture detection (GC–ECD). Geometric mean concentrations of \sum PCBs (52 congeners), \sum DDTs, \sum chlordanes, and dieldrin in all eggs were 65.0 (range=7.11 to 3930 ng/g lipid), 67.1 (range=7.88 to 1340 ng/g lipid), 37.0 (range=4.04 to 685 ng/g lipid), and 11.1 ng/g lipid (range=1.69 to 44.0 ng/g lipid), respectively. Early and middle developmental stage samples had similar concentrations of PCBs and organochlorine pesticides on a wet-mass basis (ng/g tissue extracted), but the concentrations doubled by the late stage. This increase is most likely attributable to the 50% increase in lipid content observed in the late-stage yolk. These findings indicate that an early-stage sample cannot be directly compared to a late-stage sample, especially from different nests. These preliminary findings also allowed us to calculate the minimum number of eggs per nest required for analysis to obtain an acceptable mean concentration per nest. More research is required to investigate geographical trends of contaminant concentrations and potential health effects (i.e., abnormalities) caused by these contaminants on sea turtle development.

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Keywords: Loggerhead sea turtle; Eggs; PCBs; Organochlorine pesticides; Reptile; Lipid; Embryonic development

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1. Introduction

Data on contaminant concentrations in sea turtle tissues are scarce and widely dispersed across contaminant types, geographic locations, species, and tissues (Pugh and Becker, 2001). In the last three decades, eight studies measured persistent organochlorine pollutants (POPs) in sea turtle eggs (Hillestad et al., 1974; Thompson et al., 1974; Clark and Krynnitsky, 1980; Clark and Krynnitsky, 1985; Cobb and Wood, 1997; Podreka et al., 1998; Mackenzie et al., 1999; Alam and Brim, 2000). These studies focused on loggerhead (*Caretta caretta*) and green (*Chelonia mydas*) sea turtles in the Atlantic Ocean and Mediterranean Sea. Just six of these studies report POP levels in loggerhead eggs. Most studies focused on measuring polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT) metabolites, but only three of the five PCB contributions used congener-specific analytical techniques (Cobb and Wood, 1997; Mackenzie et al., 1999; Alam and Brim, 2000).

The U.S. Department of the Interior, under the authority of the Endangered Species Act, currently lists the loggerhead sea turtle as a threatened species (Leitzell and Greenwalt, 1978). Because of their population status, it is important to measure contaminant concentrations in their tissues to better understand how chemical pollutants may impact their reproductive abilities and survival. Egg samples provide many advantages for monitoring pollutants. They can be collected in a nonlethal manner if unhatched eggs are sampled after the live hatchlings have emerged from the nest or if the chorioallantoic membranes (CAMs) can be found in remaining hatched eggshells. Loggerhead sea turtle CAMs have been validated as a nonlethal sample that represents the PCB concentrations in whole eggs (Cobb and Wood, 1997), as validated with bird and alligator CAMs as well (reviewed by Cobb et al., 2003). The drawback to using sea turtle CAMs is that this tissue can be highly disturbed in the nest chamber and mixed with beach sand during hatchling emergence. It can, however, be collected in an uncontaminated manner from late-stage embryos that fail to hatch as was done by Cobb and Wood (1997). Whether the whole egg or a portion (CAM or yolk) is sampled, unhatched eggs are fairly common, because average loggerhead turtle nests contain more than 100 eggs and hatching success is typically 80% (Miller et al., 2003). Eggs are more accessible for sampling compared to capturing juveniles or adults at sea. Moreover, the contents of sea turtle eggs represent the diet, nutrients, and chemical compounds ingested by adult females (Miller, 1997). POPs are thought to be transferred from adult females to egg yolk (Meyers-Schöne and Walton, 1994;

Mackenzie et al., 1999); therefore, the concentrations in eggs reflect the exposure not only of the developing embryo, but also of the adult females on their previous foraging grounds.

Loggerhead turtles prey on a wide range of invertebrates (i.e., crustaceans and mollusks) in benthic environments (Bjorndal, 1997). They nest every two to three years on average, each time laying up to seven clutches (Miller, 1985, 1997; Schroeder et al., 2003). Satellite tagging studies have shown that adult nesting females forage in areas far from nesting beaches. Turtles nesting on Florida's west coast migrate to resident foraging areas near Cuba, Mexico, Florida coastal waters, and in the Gulf of Mexico (Schroeder et al., 2003). Turtles nesting on the east coast of Florida forage in all of these areas as well as near the Bahamas (Schroeder et al., 2003). It is in these places that the turtles are investing energy resources into egg production (Miller et al., 2003).

The purpose of this study was to provide additional baseline data on POP concentrations in loggerhead eggs as well as to improve sampling methodologies. This study examined the change of lipid content and contaminant concentrations in the yolk through embryonic development in order to standardize future sampling efforts and analysis of additional samples. In addition, these data were also used to determine the number of egg yolk samples required per nest to provide an acceptable estimate of the average concentration per nest.

2. Materials and methods

2.1. Egg collection and processing

Eggs that failed to hatch were collected from 47 nests in collaboration with a large-scale project to evaluate sex ratios on nesting beaches along the Southeast coast of the U.S. in 2002 (Blair, 2005). Nests were not relocated (except FLBR14a), but some were caged with wire mesh to minimize predation. The egg samples analyzed in this study were selected from only three of these nests because each developmental stage was represented. Two nests were from Boca Raton, Florida (FLBR2 and FLBR14a) (near 26°23'00.9"N, 80°03'59.8"W) and one from Siesta Key near Sarasota, Florida (FLSA12) (near 27°13'7.60"N, 82°30'54.91"W). Unhatched eggs were placed in plastic bags during the excavation of each nest approximately three days to five days after the last hatchling emerged. Eggs were shipped overnight with frozen gel packs. After receipt, eggs were rinsed with deionized water to remove sand and opened to determine the stage of embryonic development at the time the embryo died, to examine for abnormalities, and

to store samples for several purposes. Only the yolk was available for contaminant analysis. The identification of early (E), middle (M), and late (L) embryonic stages were based on the following criteria: E = small white embryo, usually with eyes, without an obvious carapace; M = white embryo with a carapace, without dark scutes; L = large brown or white (amelaninic) embryo with fully formed scutes. An egg without visible development of an embryo was classified as “No” embryo. Middle to late stage embryos were measured and the gonads were stored for sexing. Yolk was separated from the albumen as much as possible and from embryos and stored frozen in hexane-rinsed aluminum foil. Yolk sacs were separated from late-stage embryos using hexane-rinsed stainless steel scissors and stored frozen in hexane-rinsed glass vials. Seven to ten egg yolk samples per nest were analyzed individually and represented one to three eggs of each stage per nest. Stages No and E were considered one group. Stages M and L were considered two separate groups. Eggs chosen from each nest are described in Table 1.

2.2. Extraction of yolk

Analysis of egg yolks for POPs followed methods modified from Kucklick et al. (2002) and Keller et al.

Table 1
Data on loggerhead sea turtle nests and egg yolks analyzed in this study

Nest ID	Egg yolk ID	Stage	SCL (mm)	Sampling date
FLBR2	FLBR2-17	No embryo	NM	July 20, 2002
	FLBR2-19	No embryo	NM	July 20, 2002
	FLBR2-27	No embryo	NM	July 20, 2002
	FLBR2-15	Middle	NM	July 20, 2002
	FLBR2-36	Middle	19.9	July 20, 2002
	FLBR2-61	Middle	16.0	July 20, 2002
	FLBR2-12	Late	33.2	July 20, 2002
	FLBR2-20	Late	NM	July 20, 2002
	FLBR2-22	Late	NM	July 20, 2002
FLBR14a	FLBR14a-19	No embryo	NM	September 25, 2002
	FLBR14a-18	Early	NM	September 25, 2002
	FLBR14a-20 ^a	Middle	NM	September 25, 2002
	FLBR14a-24	Middle	15.5	September 25, 2002
	FLBR14a-11	Late	42.8	September 25, 2002
	FLBR14a-12	Late	40.3	September 25, 2002
FLSA12	FLSA12-5	No embryo	NM	September 14, 2002
	FLSA12-8	No embryo	NM	September 14, 2002
	FLSA12-9	No embryo	NM	September 14, 2002
	FLSA12-7	Middle	11.6	September 14, 2002
	FLSA12-2	Late	32.7	September 14, 2002
	FLSA12-4	Late	29.4	September 14, 2002
	FLSA12-11	Late	40.0	September 14, 2002

FLBR = Boca Raton; Florida; FLSA = Sarasota, Florida; SCL = straight carapace length; NM = not measured.

^a Embryo with two heads.

(2004). Egg yolks (2.0 to 5.2 g) were homogenized using stainless steel spatulas in individual beakers and mixed with sodium sulfate (Na_2SO_4). Na_2SO_4 was previously combusted overnight at 700 °C to remove water and organic compounds. Homogenized samples were transferred to 33 mL pressurized fluid extractor (PFE) cells. Six calibration solutions were prepared by gravimetrically combining National Institute of Standards and Technology (NIST) Standard Reference Materials (SRMs): 2261 (Chlorinated Pesticides in Hexane), 2262 (Chlorinated Biphenyl Congeners in 2,2,4-Trimethylpentane), 2274 (PCB Congener Solution-II in Isooctane), 2275 (Chlorinated Pesticides Solution-II in Isooctane), and an additional solution containing 15 PCB congeners. The diluted calibration solutions, ranging from approximately 370 ng of each compound to 0.5 ng, were added to individual PFE cells packed with Na_2SO_4 . An internal standard solution containing 4,4'-DDT- d_8 , 4,4'-DDE- d_8 , 4,4'-DDD- d_8 , Endosulfan I- d_4 , PCB 103, and PCB 198 was added gravimetrically to all PFE cells (45 ng of each compound, 0.25 mL added). An analytical blank (a PFE cell packed with Na_2SO_4), SRM 1946 (Lake Superior Fish Tissue), and a cryohomogenized composite of ten loggerhead sea turtle egg yolks (from No/E staged eggs) from an additional nest (FLBR13) were also processed alongside the samples for quality control. Samples and calibrants were extracted with dichloromethane (DCM) in an Accelerated Solvent Extractor (Dionex, Salt Lake City, UT). The extracts of the samples, SRM 1946, and the egg composite were filtered through DCM-cleaned silicone phase separation paper (Whatman, Maidstone, England) in order to remove water. Extracts of the blank and calibration curve were not filtered because they did not contain water. Extracts were reduced to 10 mL in volume by evaporation using purified nitrogen in a Turbovap II (Zymark, Hopkinton, MA).

2.3. Lipid determination

Gravimetric lipid analysis was performed to determine the percent lipid in all yolk samples using a method modified from Keller et al. (2004). A 2 mL portion of each extract ($\approx 20\%$) was removed and transferred gravimetrically to a tared aluminum dish. The solvent was allowed to evaporate at room temperature for 6 to 12 h, and the dried lipid residue was reweighed to the nearest 0.00001 g and used to calculate the percent lipid.

2.4. Extract cleanup and analysis

Lipids and large biomolecules in the extracts were removed by size exclusion chromatography according to

Kucklick et al. (2002). Additional cleanup of the extracts was carried out using an automated solid phase extraction system (Rapid Trace SPE workstation, Zymark, Hopkinton, MA) with 1 g Florisil columns eluted with 10 mL 1:1 hexane:dichloromethane (volume fraction basis). Florisil was previously combusted at 700 °C, and 12 µL of water was added to each g of Florisil just prior to use. The samples were fractionated with silica columns to separate the lower polarity compounds resulting in fraction 1 (F1) from the higher polarity compounds in fraction 2 (F2). Compounds contained in F1 included 52 PCB congeners, heptachlor, 2,4'-DDE, 4,4'-DDE, 2,4'-DDT, hexachlorobenzene (HCB), aldrin, and mirex. Analytes in F2 included 4,4'-DDT, *cis*- and *trans*-chlordane, *cis*- and *trans*-nonachlor, α - β -, and γ -hexachlorocyclohexane (HCH), heptachlor epoxide, oxychlordane, 2,4'-DDD, 4,4'-DDD, dieldrin, endrin, endosulfan I and II, and endosulfan sulfate. Contaminant concentrations were determined by gas chromatography with electron capture detection (GC–ECD) according to Kucklick et al. (2002). The amount of each compound was calculated using the slope and intercept of linear regressions (amount and peak area ratios of the analyte of interest to the internal standards as *x*-axis and *y*-axis variables, respectively) containing at least three calibration points. The limits of detection (LODs) were determined by integrating the noise in the blank for each compound and calculating the nanograms of each compound in the blank using the calibration curve (if a negative value was obtained, the *y*-intercept was set to zero). The LOD was established for each sample and each compound as 3 times the nanograms calculated in the blank divided by the sample mass extracted.

2.5. Statistical analysis

Statistical analyses were conducted using JMP 5.1 (SAS, Institute Inc., Cary, NC). Σ PCBs were defined as the sum of 29 detected individual PCB congeners. Σ DDT was defined as the sum of 2,4'-DDE, 4,4'-DDE, and 4,4'-DDD, and Σ chlordanes as the sum of heptachlor epoxide, oxychlordane, *cis*-chlordane, *cis*-nonachlor, and *trans*-nonachlor. Additional PCB congeners and pesticides were not included because they were not detected in the samples. Organochlorine contaminant concentrations were lipid normalized by dividing the wet-mass concentration by the fraction of lipid. Lipid-normalized POP concentrations were normally distributed within each nest as tested by the Shapiro–Wilk *W* test. Differences in concentrations among nests were determined using a Welch analysis of variance (ANOVA), because the variances of each nest were

unequal (Bartlett test $p < 0.05$). A Turkey–Kramer multiple comparison test was used to determine which nests were different from the others. To examine changes in contaminant concentrations and lipid content among developmental stages, the percent change from the No/E stage was calculated for the M and L stages of each nest. The percent changes were averaged across the three nests, and these were normally distributed (Shapiro–Wilk *W* test, $p > 0.05$) with equal variances between the M and L stages (Bartlett test, $p > 0.05$). The Student's *t*-test was used to determine differences in contaminant concentrations and lipid content between middle and late stages of development. Pearson correlations were used to examine the relationship between percent lipid and straight carapace length (SCL). Iterations of the following equation were used to estimate the required number of eggs (*n*) from each nest to obtain an average concentration for that nest that was within an acceptable confidence interval of the measured nest mean concentration:

$$n = \frac{SD^2 * t_{0.05(2),df}^2}{d^2}$$

where SD = standard deviation; *t* = two-tailed critical value of a Student's *t*-test with $\alpha = 0.05$; *df* = degrees of freedom or (*n* – 1); *d* = half of the desired confidence interval, which was chosen as 30%, 20%, or 10% above or below the measured mean concentration (Zar, 1999). This parametric analysis, performed on each nest individually and then averaged across nests, was possible because the POP concentrations were normally distributed within each nest (Shapiro–Wilk *W* test, $p > 0.05$).

3. Results

3.1. Quality control analysis

The PCB and pesticide concentrations measured in two replicates of SRM 1946 differed from NIST's certified values by an average of –6.8% (range = –27% to 17%) (Table 2), which demonstrates acceptable accuracy. The lipid content measured in SRM 1946 was within 17% of the certified values (Table 2). Concentrations measured in two replicates of a loggerhead egg composite control material demonstrated precision even at low concentrations (Table 2). The majority of POPs were found below 0.200 ng in the blank sample, and the blank had concentrations that ranged from 0.020 ng for PCB 206 to 11.5 ng for 4,4'-DDD.

Table 2

Concentrations of selected PCBs and pesticides (ng/g wet mass) measured in two aliquots of National Institute of Standards and Technology Standard Reference Material 1946 (Lake Superior Fish Tissue) compared to certified concentrations and two aliquots of a loggerhead egg composite control material

Compound	SRM 1946		Loggerhead egg composite		
	Certified value (ng/g wet mass)	Measured concentration (ng/g wet mass)		Measured concentration (ng/g wet mass)	
		Rep 1 ^a	Rep 2	Rep 1 ^a	Rep 2
PCB 99	25.6±2.3	26.5	23.5	1.79	1.90
PCB 105	19.9±0.9	17.7	19.0	0.488	0.574
PCB 118	52.1±1.0	48.3	48.2	1.31	1.22
PCB 128	22.8±1.9	20.4	21.7	0.495	0.575
PCB 138+163	115±13 ^b	123	135	2.85	3.57
PCB 146	30.1±3.5	22.1	22.2	0.515	0.726
PCB 153	170±9	161	155	4.46	6.39
PCB 170	25.2±2.2	25.9	22.3	0.465	0.492
PCB 180	74.4±4.0	72.9	71.3	1.10	1.18
PCB 187	55.2±2.1	51.6	47.0	1.23	1.23
Mirex	6.47±0.77	5.63	5.83	0.231	0.185
Dieldrin	32.5±3.5	29.8	30.4	1.21	0.964
Heptachlor epoxide	5.50±0.23	5.79	6.17	0.235	0.178
Oxychlorodane	18.9±1.5	15.1	18.2	<LOD ^c	0.185
<i>trans</i> -nonachlor	99.6±7.6	87.6	79.7	1.34	1.31
4,4'-DDE	373±48	354	351	6.73	7.92
Percent lipid (%)	10.17±0.48	8.45	9.85	8.48	8.31

Certified values taken from the Certificate of Analysis for SRM 1946.

^a Replicate 1 was analyzed with the samples reported in this study; replicate 2 was analyzed the following year by the same personnel with similar methods.

^b Certified value for only PCB 138.

^c Measured below the limit of detection, which was 0.120 ng/g wet mass for oxychlorodane in this sample.

3.2. Lipid content

The percent lipid in all egg yolk samples averaged 15.0% with a standard deviation of 4.7% and a range of 7.70% to 25.6%. No significant difference in yolk lipid

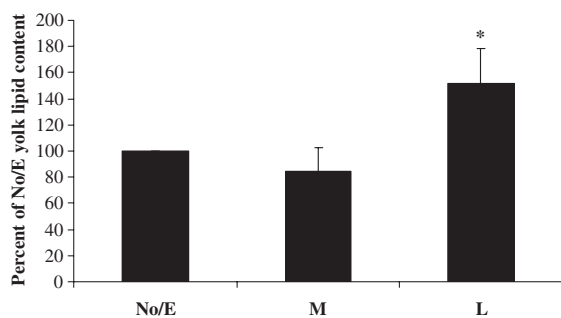


Fig. 1. Percent change in lipid content of loggerhead sea turtle yolk samples through embryonic development from no development/early (No/E) to middle (M) and late (L) stages. Percent change was calculated relative to the average lipid content of the No/E stage of each nest. Mean and one standard deviation (error bars) were calculated from three nests. The asterisk indicates a significant difference between the M and L stages (Student's *t*-test; $p=0.015$).

content was observed among nests when all developmental stages were averaged per nest (ANOVA; $p=0.986$; data not shown). Yolk lipid content (expressed relative to the percent lipid in the No/E stage within each nest) significantly increased between the middle and late stages of development (Student's *t*-test; $p=0.015$; Fig. 1). Percent lipid in yolk sacs of late-stage embryos was approximately 1.5 fold higher than earlier stages (No/E

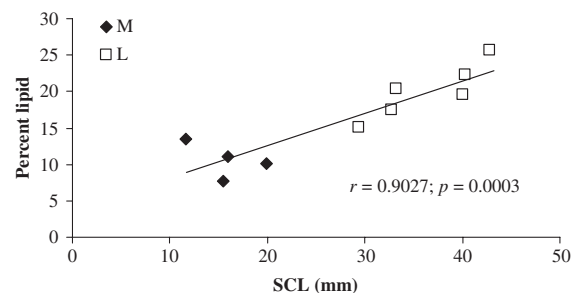


Fig. 2. Percent lipid in yolk versus straight carapace length (SCL) of ten loggerhead embryos of middle (M) and late (L) developmental stages from three nests. Linear trendline and Pearson correlation statistics indicate a strong positive correlation.

Table 3
Lipid-normalized concentrations (ng/g lipid) of POPs in loggerhead egg yolk samples ($n=22$ eggs from 3 nests)

Compound	Geometric mean ^a (ng/g lipid)	Arith. mean ^a ±SD (ng/g lipid)	Range ^a (ng/g lipid)	#>LOD ^b
Total PCBs	65.0	904±1500	7.11–3930	22
2,4'-DDE	1.64	3.30±4.11	0.514–11.3	6
4,4'-DDE	66.9	314±485	7.24–1330	22
4,4'-DDD	0.308	0.417±0.415	0.122–1.72	4
Total DDTs	67.1	318±485	7.88–1340	22
<i>trans</i> -nonachlor	12.3	94.1±153	1.40–430	22
Oxychlordane	8.97	52.3±83.0	0.981–217	22
Heptachlor epoxide	7.43	11.7±10.4	1.32–30.8	22
<i>cis</i> -nonachlor	0.815	1.73±1.98	0.095–5.56	17
<i>cis</i> -chlordane	0.631	0.715±0.364	0.243–1.70	15
Total chlordanes	37.0	161±249	4.04–685	22
Dieldrin	11.1	16.1±13.0	1.69–44.0	22
Mirex	0.410	6.41±10.7	0.057–29.4	6
α-HCH	0.458	0.985±1.28	0.131–3.82	6
β-HCH	0.550	1.06±1.32	0.172–4.70	6
Total POPs	212	1400±2250	22.2–6000	22

SD = standard deviation; LOD = limit of detection.

^a Sample concentrations <LOD were set to zero in order to calculate totals. For compounds shown as individuals, concentrations <LOD were set to a random value between half the LOD and the LOD.

^b The number of eggs out of 22 that had detectable concentrations.

and M). For the middle and late stages, yolk lipid content was significantly correlated with SCL of the embryo ($r=0.9027$; $p=0.0003$; Fig. 2).

3.3. POP concentrations and patterns

Average lipid-normalized concentrations of Σ PCBs and organochlorine pesticides for all yolk samples are presented in Table 3. PCBs were the predominant organochlorine compound class, representing 65% of the Σ POP concentration. Of the 52 targeted PCB congeners, 29 were present in at least one egg sample. PCB congeners 153, 138+163, and 118 were the most abundant (Fig. 3) and were present at mean concentrations above 100 ng/g lipid. PCB 153 was the most predominant congener in all samples, accounting for 38% of the mean Σ PCB concentration (Fig. 3). Penta-, hexa-, and heptachlorobiphenyl homologue groups represented the majority of the Σ PCB concentration.

DDTs were the second most abundant POP class measured, mainly represented by the major metabolite, 4,4'-DDE, which represented 99% of the Σ DDT concentration (Table 3). Σ DDTs represented 23% of the Σ POP concentration. Chlordane pesticides were the third most abundant group of POPs representing 11.5% of Σ POP concentrations. *trans*-nonachlor contributed the highest concentration and represented 58% of the Σ chlordanes, whereas oxychlordane represented 32% of the Σ chlordanes concentration. The organochlorine pesticides, α-HCH, β-HCH, mirex, and dieldrin, combined, represented 1.7% of the Σ POP concentration. Concentrations of the organochlorine pesticides, endosulfan I and II, endosulfan sulfate, and HCB, were not detected in any of the egg samples.

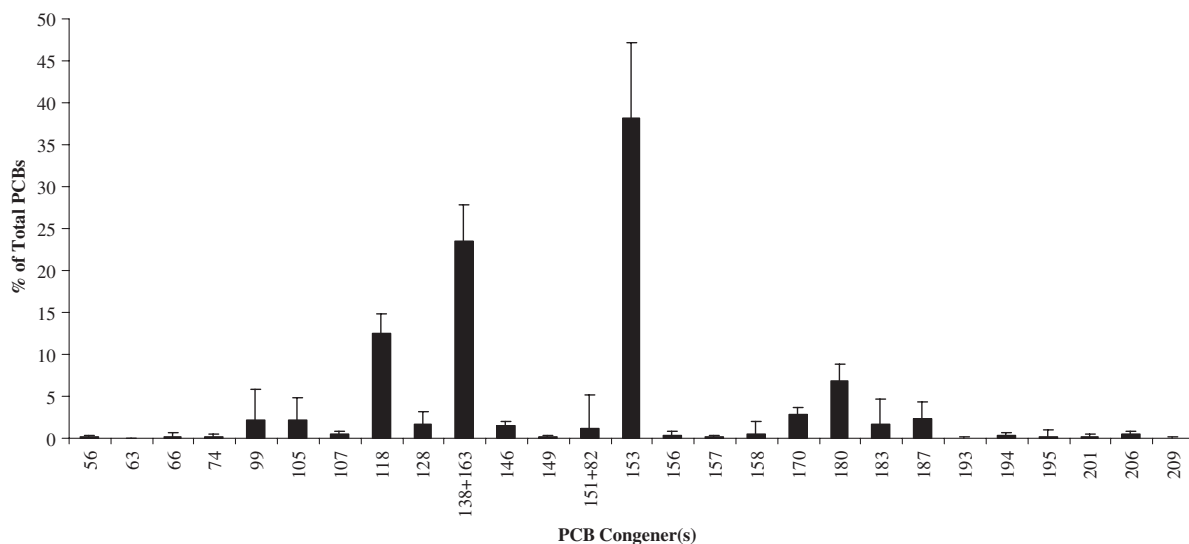


Fig. 3. Contribution of individual PCB congeners to the Σ PCB concentration on a lipid-normalized basis from all loggerhead sea turtle egg yolk samples from three nests. Percentages were calculated for each individual sample and all samples were averaged. Error bars are one standard deviation.

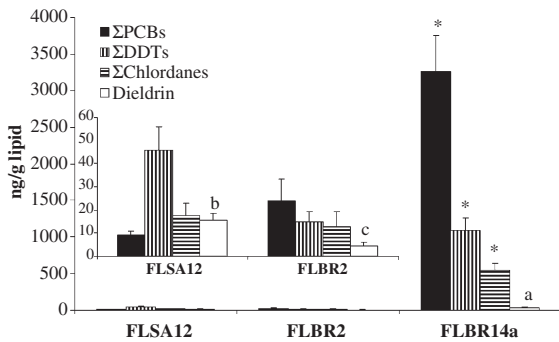


Fig. 4. Comparison of Σ PCB, Σ DDT, Σ chlordanes and dieldrin concentrations among three loggerhead sea turtle nests. An asterisk indicates a significant difference in concentrations from the other two nests; different letters indicate significant differences among nests (Welch ANOVA $p < 0.0001$ with Tukey–Kramer multiple comparisons test $p < 0.05$). Error bars are one standard deviation.

3.4. Differences in POP concentrations among nests

Lipid-normalized concentrations of Σ PCBs, Σ DDTs, Σ chlordanes, and dieldrin differed among the three nests (Welch ANOVA, $p < 0.0001$; Fig. 4). Egg samples from nest FLBR14a had the highest concentrations of all of these compounds. The less predominant pesticides, mirex, α -HCH, and β -HCH, were detectable only in FLBR14a. The pattern of POP classes was similar between the two Boca Raton nests with the Σ PCBs occurring at the highest concentrations followed by Σ DDTs, Σ chlordanes, and dieldrin. However, the pattern observed in the nest from the west coast of Florida (FLSA12) was different. In FLSA12, Σ DDTs (45.9 ng/g lipid) dominated the pattern, followed by Σ chlordanes (17.4 ng/g lipid), dieldrin (15.5 ng/g lipid) and Σ PCBs (9.21 ng/g lipid).

3.5. Changes in POP concentrations through developmental stages

To examine changes in POP concentrations through development, the average concentrations for each stage within each nest were expressed as a percent of the average concentration in the No/E stage of that nest. The differences among developmental stages were stronger when the POP concentrations were reported on a wet-mass basis (Fig. 5a) compared to a lipid-normalized basis (Fig. 5b). On a wet-mass basis, the late-stage egg yolks had approximately double the concentrations of Σ PCBs, Σ DDTs, Σ chlordanes, and dieldrin compared to the No/E and M stages (Fig. 5a). The late-stage concentrations were significantly higher than the middle stage (Student's t -test, $p < 0.05$). On a lipid-normalized

basis, the percent change from the No/E concentrations did not differ among developmental stages for Σ PCBs, Σ DDTs, or dieldrin ($p > 0.05$), but Σ chlordanes significantly increased at the late stage by 35% ($p < 0.05$) (Fig. 5b).

3.6. Sample size

Based on the observed change in POP concentrations detected in the late-stage samples, only No, E, and M stages from each nest were used in this statistical analysis. The number of eggs required per nest to yield an acceptable average estimate of Σ POP concentrations is presented in Table 4. For Σ POP concentrations, a respective sample size of approximately 3, 4, or 11 eggs per nest would be required to obtain an average nest concentration that was within 30%, 20%, or 10% of the measured sample mean. If concentrations of late-stage eggs are incorporated in the estimation, a sample size of

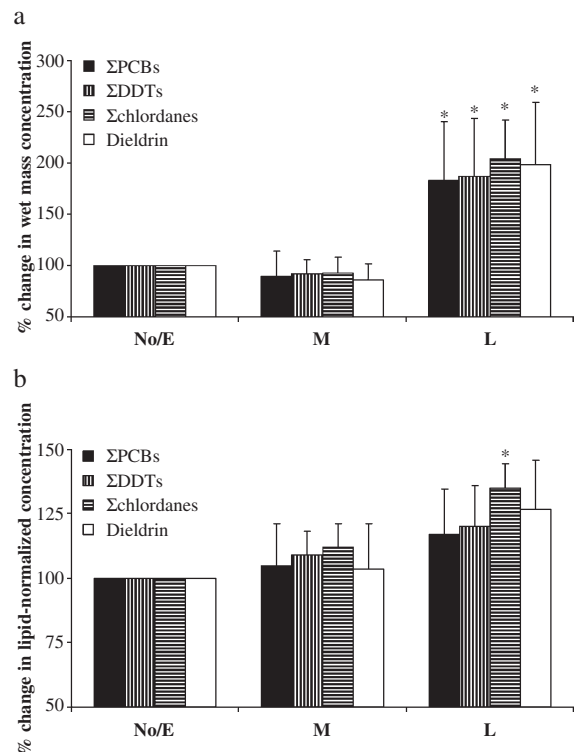


Fig. 5. Percent change in POP concentrations in loggerhead egg yolk samples through development from no development/early (No/E) to middle (M) and late (L) stages on a) a wet-mass basis and b) on a lipid-normalized basis. Percent change was calculated relative to the average concentrations of the No/E stage of each nest. Mean and one standard deviation (error bars) were calculated from three nests. An asterisk indicates a significant difference between the M and L stage concentrations (Student's t -test; $p < 0.05$).

Table 4

Minimum loggerhead egg sample size required per nest to obtain an acceptable estimate of lipid-normalized POP concentrations for each nest based on eggs with no development, early-stage, or middle-stage embryos

	Predicted n to achieve a population mean within		
	30% of sample mean	20% of sample mean	10% of sample mean
Σ PCBs	4	6	17
Σ DDTs	2	4	9
Σ chlordanes	6	10	33
Dieldrin	3	4	11
Σ POPs	3	4	11

n = sample size.

Predicted n was derived from the following equation: $n = (SD^2)(t^2_{0.05(2),df})/(\bar{d}^2)$ where SD = standard deviation, t = two-tailed critical value of a Student's t -test with $\alpha = 0.05$; df = degrees of freedom or $(n - 1)$; d = half of the desired confidence interval, which was chosen here as 30%, 20%, or 10% above or below the measured mean concentration (Zar, 1999). The average of the derived n for three nests is shown here.

more than 9 eggs per nest would be required to be within 20% of the measured sample mean Σ POPs.

4. Discussion

This study provides additional baseline data on contaminant concentrations in sea turtle eggs. POP concentrations (ng/g wet mass) measured in this study are compared to previous studies that analyzed sea turtle eggs in Table 5. One previous study was not included in the table because it reported concentrations only on a lipid-normalized basis (Cobb and Wood, 1997). The average Σ PCB concentration (904 ng/g lipid) measured in the current study was similar to that found in loggerhead eggs (1188 ng/g lipid) from Cape Island, South Carolina (Cobb and Wood, 1997). A decline in POP concentrations is not evident based on a comparison of the present study to those performed in the 1970s and 1980s along the Southeast coast of the U.S. (Hillestad et al., 1974; Clark and Krynitsky, 1980, 1985 in Table 5). In fact, PCB and chlordanes concentrations appear to have increased, while DDT concentrations have remained relatively constant. These comparisons suggest that most POP concentrations have not substantially decreased through time, even though most of these compounds were banned from use in the U.S. and other developed countries. This conclusion, however, must be considered with caution, because a long-term monitoring study has observed declining trends in PCB and pesticide concentrations in Atlantic menhaden (*Brevoortia tyrannus*) from this region (Mitchum, unpublished data). Additionally, the temporal comparison of concentrations in

sea turtles is weakened by their migratory behavior, which may cause them to reflect exposure levels from other countries where these compounds are still being used. In addition, the comparison among the present and previous studies is limited, because different analytical methodologies, sample sizes, and sampling locations were used.

The POP concentrations measured in the loggerhead egg samples in the present study are higher than those measured previously in green sea turtle eggs (Table 5). For example, the mean 4,4'-DDE concentration in the loggerhead eggs was more than ten times higher than the concentrations found in green turtle eggs from four locations (Table 5). These species differences have been observed previously and have been explained by differing trophic status (Meyers-Schöne and Walton, 1994). Green turtles are herbivores, so they do not accumulate POPs to the same level as omnivorous loggerhead turtles.

POP concentrations in eggs from other reptile species are generally higher than those measured in loggerhead eggs. For example, Σ PCB concentrations in the loggerhead eggs (144 ng/g wet mass) were lower than the average Σ PCB concentrations measured in snapping turtle (*Chelydra serpentina*) eggs from several sites in the Great Lakes (241 ng/g wet mass to 3946 ng/g wet mass), except for a reference site (17.9 ng/g wet mass) (Bishop et al., 1998). Similarly, the loggerhead eggs had lower concentrations of 4,4'-DDE (49.7 ng/g wet mass) compared to *Alligator mississippiensis* (range: 100 to 7600 ng/g wet mass) (Heinz et al., 1991) and *Crocodylus acutus* (range: 370 to 2900 ng/g wet mass) (Hall et al., 1979). On the other hand, the maximum concentration of Σ PCBs measured here (1010 ng/g wet mass) was greater than the maximum found in eggs of *A. mississippiensis* from Lakes Apopka (450 ng/g wet mass) and Lake Griffin (670 ng/g wet mass) in Florida (Heinz et al., 1991). The mean Σ PCB concentration measured in the current study (904 ng/g lipid) was bracketed by concentrations measured in egg contents of *A. mississippiensis* collected from two sites in South Carolina (333 ng/g lipid and 3176 ng/g lipid) and was higher than alligator eggs collected from Louisiana (218 ng/g lipid) (Cobb et al., 2002). The geometric mean of 4,4'-DDE measured in Morelet's crocodile eggs from Belize (48.0 ng/g wet mass) (Wu et al., 2000) was higher than that in the current study (9.60 ng/g wet mass). These different levels of exposure are most likely due to differences in proximity to contaminant sources and/or differences in trophic levels.

Two nests from the same beach (Boca Raton, FL) had very different POP concentrations, but they had similar

Table 5

Comparison of measured organochlorine concentrations, mean (SD) [range], in ng/g wet mass in eggs of sea turtles

Species	<i>n</i> ^a	Location	∑PCBs	∑DDTs	∑chlordanes	Dieldrin	Mirex	∑HCH	Reference
<i>C. caretta</i>	22 (3)	Boca Raton and Sarasota, Florida	144 (280) [0.796–1010]	50.2 (92.4) [1.19–344]	25.5 (46.7) [0.827–175]	2.53 (2.76) [0.278–10.8]	1.03 (2.04) [0.009–7.52]	0.258 (0.508) [0.020–2.05] ^b	This study
<i>C. caretta</i>	NR	S. Carolina and Georgia	NM	[58–305] ^c	NM	ND–56.4	NM	NM	Hillestad et al. (1974)
<i>C. caretta</i>	9 (9)	Merritt Island, Florida	78 ^d [32–201]	66 ^e [18–200]	[ND–6, 17, or 9] ^f	ND	[ND–5]	NM	Clark and Krynitsky (1980)
<i>C. caretta</i>	56 (1)	Merritt Island, Florida	NM	99 ^g [56.0–150]	[ND–8] ^h	NM	NM	NM	Clark and Krynitsky (1985)
<i>C. caretta</i>	1	Cyprus, Mediterranean Sea	89	155 ⁱ	1.8 ^j	0.6	NM	NM	Mackenzie et al. (1999)
<i>C. caretta</i>	4–12 eggs pooled/ nest (20)	Northwest Florida	[240–3720] ^k	[ND–178] ^{k,1}	ND	ND	ND	ND	Alam and Brim (2000)
<i>C. mydas</i>	2 (2)	Merritt Island, Florida	ND	2 ^e [ND–5]	ND	ND	ND	NM	Clark and Krynitsky (1980)
<i>C. mydas</i>	10 (4)	Ascension Island	76 (64) [20.0–220] ^m	3 (3) [ND–9.00] ^e	NM	NM	NM	NM	Thompson et al. (1974)
<i>C. mydas</i>	15 (4)	Heron Island, Queensland, Australia	NM	1.7 [1.3–2.4] ^e	NM	NM	NM	NM	Podreka et al. (1998)
<i>C. mydas</i>	1	Cyprus, Mediterranean Sea	6.1	4.3 ⁱ	< 0.3	< 0.3	NM	NM	Mackenzie et al. (1999)

C. caretta: loggerhead sea turtle; *C. mydas*: green sea turtle; NR: not reported; NM: not measured; ND: not detected because value was below the detection limit.

^a *n* = total number of eggs analyzed individually from a total number of nests in parentheses.

^b Sum of α-HCH and β-HCH.

^c Sum of “DDE, DDD, and DDT”.

^d Geometric mean; total PCBs were measured as Aroclor 1260.

^e Arithmetic mean for only 4,4'-DDE.

^f Range of heptachlor epoxide, oxychlorodane, and *trans*-nonachlor, respectively.

^g Geometric mean for only 4,4'-DDE.

^h Approximate range for *cis*-chlordanes and oxychlorodane, individually; both had the same range.

ⁱ Sum of 4,4'-DDE, 4,4'-DDD, and 4,4'-DDT.

^j Sum of heptachlor, heptachlor epoxide, α-chlordanes, γ-chlordanes, oxychlorodane, and *trans*-nonachlor.

^k Values converted from ng/g dry mass to ng/g wet mass.

¹ Reported as 4,4'-DDD alone.

^m Total PCBs were measured as Aroclor 1242 or 1248.

patterns (i.e., ∑PCBs > ∑DDTs > ∑chlordanes). The nest laid near Sarasota on the west coast of FL (FLSA12) had similar concentrations compared to the less contaminated nest from Boca Raton, but it contained a unique pattern in which DDTs were more abundant than PCBs. This pattern suggests that the adult female that laid nest FLSA12 was foraging in areas with relatively more contamination by organochlorine pesticides compared to PCBs. These similarities and differences highlight the hypothesis that adult foraging

sites, far from the nesting beach, greatly influence the levels and patterns transferred to eggs. Interestingly, the highest levels of POPs were found in nest FLBR14a (mean ∑POP = 4940 ng/g lipid), which contained a two-headed embryo. Abnormality rates have been shown to increase in snapping turtle hatchlings from the Great Lakes in relation to higher egg concentrations of chlorinated contaminants (Bishop et al., 1998). These findings emphasize the need to examine geographical differences in contamination of loggerhead eggs and

the potential developmental effects caused by these contaminants.

This study compared organochlorine contaminants in yolk among developmental stages in loggerhead eggs in order to develop standardized sampling methods for long-term monitoring. This was important for two reasons. Firstly, this study was part of a larger project investigating the sex ratio of loggerhead turtles hatching on nesting beaches located throughout the southeastern coast of the U.S. (Blair, 2005). Many sea turtle studies, such as this one, involve numerous collaborators, resulting in the need to subdivide samples for different purposes. Only the egg yolk was available for contaminant measurements in this study, but the eggs represented a variety of developmental stages. Prior to analyzing the remaining 44 nests, we needed to determine if any stage could be analyzed or if one stage should be the focus.

A related but more broadly important reason for this study is that the yolk sac visibly changes throughout development of the embryo. It becomes smaller and more viscous in late stages. During the last stage of embryonic development (final third of development) the yolk volume decreases to approximately 50% of the volume of the embryo, and during pipping the remaining yolk sac is completely internalized into the body cavity (Miller et al., 2003). Hatchlings require this high-energy source for the first few days of frenzy swimming. In order to reduce the size of the yolk sac, we hypothesized that some component of the yolk, probably water, is removed and utilized while lipids are concentrated. A portion of this hypothesis was supported by the finding that lipid content increased by 50% between the middle and late stages. A similar increase in yolk lipid content was noted previously for three other reptile species (Rowe et al., 1995). This process of concentrating the lipids also appeared to increase the concentration of hydrophobic POPs. The yolk of the late-stage embryos analyzed in the current study had POP concentrations, on a wet-mass basis, that were approximately two-fold greater than earlier stages. Similar findings have been observed in the yolk plus albumen portion of white leghorn chicken (*Gallus domesticus*) eggs after exposing hens to a mixture of three PCBs and endosulfan (Bargar et al., 2001). In that study, lipid content steadily increased through embryonic development, and PCB concentrations on a wet-mass basis increased five-fold from day 9 to day 19 of incubation.

Changes in POP concentrations through development were larger when the data were reported on a wet-mass basis compared to lipid-normalized concentrations. This finding provides more evidence that the POPs are being concentrated along with the lipids and is important for

two reasons. Methodologically, yolk samples from earlier stages from one nest cannot be directly compared to samples from late stages, especially from different nests. Future studies that utilize only a portion of the egg contents should focus consistently on either the earlier or the later developmental stages. If different stages are used, it is important to normalize contaminant concentrations to the lipid content measured in the samples. Another important implication is that rapid mobilization of these concentrated lipids during the first few days after hatching will increase the exposure of target tissues to POPs at this vulnerable life stage. Future studies should investigate the toxic effects of hydrophobic contaminants on developing sea turtles, especially during the early post-hatchling stage.

Sample size is a major aspect in designing experiments and monitoring programs. Larger sample sizes are advantageous for statistical testing, but field sampling is often limited by availability of funds and time and by the species under study (i.e., protected species). As shown in Table 4, the number of eggs per nest required for Σ POP analysis increases to 11 as the allowable deviation from the true nest mean decreases to 10% versus only 3 eggs required for a 30% deviation from the mean. The probability of obtaining 3 eggs of these developmental stages per loggerhead nest is high. For example, 37 of the 47 nests (79%) sampled in the larger portion of this study had 3 or more unhatched eggs of these earlier developmental stages. The coefficient of variation of POP concentrations within nests considering only the No/E and middle stages is relatively small (<20%). The late stage was excluded from the calculation of the sample size requirements since this stage exhibited an increase in wet-mass POP concentrations, resulting in increased variability within each nest and the need to analyze more eggs per nest.

5. Conclusion

This study provides a broader foundation for future research and monitoring of sea turtle eggs for contaminant concentrations and their toxic effects. We conclude two important findings that will be utilized when we expand our analytical efforts and should be considered for all future sampling efforts. Firstly, No/E and M stages cannot be directly compared to L stages, especially from different nests. And, three or more eggs per nest are necessary to obtain acceptable average nest concentrations of Σ POPs. Future studies will evaluate geographical trends along the Southeast coast of the U.S. and the relationships of these compounds to hatching success, embryonic abnormality rates,

hatchling survival rates, sex ratios, and hatchling growth rates.

Acknowledgements

The authors thank Stacy Vander Pol (NIST) and Ryan Templeton (Research Experience for Undergraduates) for their technical assistance, Allan Strand (College of Charleston) for statistical advice, and everyone who participated in egg collection during the sex ratio project, including Jesse Marsh, Catherine McClellan, Matt Rush, Kim Blair, Lesley Stokes, Kelly Stewart, Matthew Godfrey, Jeff Cordes, Jerris Foote, Kirt Rusenko, Chris Johnson, Michael Bresette, Rick Herren, Stacy Kubis, Kelly Roberts, and Dean Bagley from Duke University, Florida Atlantic University, North Carolina Wildlife Resources Commission, Cape Lookout National Seashore, Mote Marine Laboratory, Gumbo Limbo Nature Center, Marinelife Center of Juno Beach, Quantum Resources at the St. Lucie Power Plant, and University of Central Florida. The sex ratio project was funded by the U.S. Environmental Protection Agency STAR grant R82-9094 (JW, LC). Academic support and scholarship for JJA was granted by the Fulbright-Organization of American States (OAS) Ecology Program through the Fulbright Commission/Foundation, the Academic and Professional Programs for the Americas (LASPAU), and the OAS. Additional funding and support for JJA was provided by the Center for Coastal Environmental Health and Biomolecular Research, the National Oceanographic and Atmospheric Agency, and the National Ocean Service.

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