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The distribution and stratification of persistent organic pollutants and fatty acids in bottlenose dolphin (*Tursiops truncatus*) blubber

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HIGHLIGHTS

- We examined pollutants (POPs) and fatty acids (FA) in dolphin blubber to assess stratification.
- Dietary and biosynthesized FAs stratified reflecting recent feeding or storage.
- Total POPs were stratified but the stratification direction varied among individuals.
- Profiles of individual POPs were consistent among blubber layers and body locations.
- POP assessment in partial depth biopsies can be used however variability will be greater.

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ABSTRACT

Blubber has been used for decades to monitor exposure of marine mammals to persistent organic pollutants (POPs). However, little is known about POP variability as a function of blubber depth and across the body of the animal. Remote blubber biopsy sampling (e.g, projectile biopsy) is the most common technique used to acquire samples from free-swimming animals, yet such techniques may result in variable sampling. It is important to understand whether blubber stratification or body location affects POP concentration or the concentration of other important blubber constituents such as fatty acids (FA). To investigate the influence of sampling depth and location on POP concentration, full depth blubber samples were taken from one stranded bottlenose dolphin (*Tursiops truncatus*) at six different body sites to assess variation in FA distribution and contaminant storage with body location. Three of the samples from different body locations were separated into histologically distinct layers to examine the effect of blubber depth and body location on POPs and FAs. In this individual, both POPs and FAs were heterogeneous with blubber depth and body location. POP concentrations were significantly greater in ventral (average ΣPBDEs 1350 ng/g lipid) and anterior (average ΣPCBs 28700 ng/g lipid) body locations and greater in the superficial blubber layer (average ΣPCBs 35500 ng/g lipid) when compared to the deep (8390 ng/g lipid) and middle (23,700 ng/g lipid) layers. Proportionally more dietary FAs were found in dorsal blubber and in middle and deep layers relative to other locations while the reverse was true for biosynthesized FAs. Stratification was further examined in blubber from the same body location in five additional stranded bottlenose dolphins. Although FAs were stratified with blubber depth, lipid-normalized POPs were not significantly different with depth, indicating that POP concentrations can vary in an individual with blubber depth though the direction of POP stratification is not consistent among individuals.

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1. Introduction

The marine environment is a global sink for lipophilic persistent organic pollutants (POPs) that are legacies of prior use (e.g, organochlorine pesticides, polychlorinated biphenyls) or current domestic use (e.g, brominated flame retardants) (Dachs et al., 2002). Due to their resistance to degradation and lipophilic properties, POPs accumulate in the lipid-rich tissues of animals, such as the blubber of cetaceans, where they can have significant negative effects on animal health and reproductive ability (Beland et al., 1993; Schwacke et al., 2011; Wells et al., 2005).

Samples from odontocetes (toothed whales) have been used to gauge POP contamination in the marine environment due to the high trophic positions of these animals and often close association

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with coastal areas where sampling is easier to conduct (Kucklick et al., 2011). Non-lethal remote biopsy sampling of blubber has become a common method for sampling odontocetes, rather than sampling blubber from stranded or fisheries bycatch animals. POP concentrations in blubber samples obtained from free ranging animals are preferable to those obtained from stranded animals as the habitat use history and health status of stranded animals are generally difficult to determine. Conversely, results obtained from living animals can sometimes be coupled to known habitat preference or association with contaminated sites (Balmer et al., 2011; Fair et al., 2007; Kucklick et al., 2011; Litz et al., 2007; Pulster et al., 2009).

While samples from remotely collected biopsies provide important information on POP exposure in odontocetes, it is recognized that blubber tissue is not homogeneous with depth or across the body (Evans et al., 2004; Montie et al., 2008; Struntz et al., 2004). Further, the influence of this variability on POP distribution in small odontocetes (i.e., the bottlenose dolphin (*Tursiops truncatus*)) that are most commonly sampled is largely unknown. This uncertainty can lead to difficulty in interpreting POP and/or fatty acid (FA) data when the biopsy sample does not penetrate the entire blubber layer during collection. Sampling the same location on different free-swimming animals using a projectile biopsy is also difficult.

In the bottlenose dolphin, adipocyte dimensions, vascularization, and distribution of structural collagen fibers in blubber has been shown to vary with depth (McClelland et al., 2012; Pond, 1978; Struntz et al., 2004) with superficial, middle and deep layers being identified. Within the depth of the bottlenose dolphin blubber, the middle and deep layers, those closest to the muscle interface, appear to be the most metabolically dynamic serving as the main location for accumulation and release of nutritional lipids (Struntz et al., 2004). Likewise, blubber composition varies with body location. For example, in bottlenose dolphins the tailstock blubber is thought to serve a mainly locomotor function and is not a physiologically active site for sequestration or release of energy (Pabst, 1996), while thoracic blubber is the main region associated with energy storage and release. Specific changes in blubber composition have also been observed with age, reproductive status, season, and geographic location (Dunkin et al., 2005; Samuel and Worthy, 2004; Struntz et al., 2004).

FA stratification has been observed in bottlenose dolphin blubber. Higher concentrations of biosynthesized and branched chain FAs are generally more abundant in the superficial layer, higher concentrations of dietary FAs are found in deep blubber, and intermediate layers have characteristics of both superficial and deep layers (Samuel and Worthy, 2004), suggesting that lipid mobilization and deposition is most active in the deep blubber (Koopman et al., 1996). Although FA composition does vary with blubber depth, it has not been shown to vary at different body sites in the same dolphin (Samuel and Worthy, 2004).

Struntz et al. (2004) hypothesized that the deep adipocytes of juvenile bottlenose dolphins may be preferentially filled with lipid from dietary sources, suggesting that contaminants in association with dietary lipids would also be deposited in the deep blubber layers. It is not known, however, if contaminants remain in the deep blubber layer with the possibility of re-entering the bloodstream during lipid catabolism or if they are permanently retained in adipocytes in the superficial blubber layer. Recent data suggest the former and that the expression of CYP1A, an enzyme involved in xenobiotic metabolism, is heterogeneously distributed in the blubber, with higher expression in the deep layer compared to superficial layers, implying POP metabolism occurs to a greater extent in this deep layer (Montie et al., 2008). Few studies have assessed POP stratification with blubber depth in small odontocetes; however, several studies have been conducted using larger whales, though POPs were found to be homogenously distributed (Evans et al., 2004). It is likely that POPs are in a dynamic equilibrium with adipocytes and other tissues and that this equilibrium is a function of lipid content (Yordy et al., 2010a).

The aim of this study was to examine the relationship of POP and FA distributions with body location and blubber depth in order to better understand how POPs are arranged in bottlenose dolphin blubber and provide insight on bottlenose dolphin exposure to labile fat-soluble contaminants. In addition, this study aims to provide a better understanding of how to interpret POP data from blubber samples obtained from different body locations and blubber depths.

2. Materials and Methods

2.1. Sample Collection

Blubber samples were collected from six subadult male bottlenose dolphins that were either stranded or were incidentally killed in fishing operations near the North Carolina and Virginia coasts (Table 1). Subadult (i.e. reproductively immature) animals were classified based on total body length and testicular measurements. Only animals that were code 1 or code 2 (euthanized or freshly dead, respectively) were used (Hofman, 1991). Only subadult males were chosen in order to minimize variation between individuals due to life-history characteristics and state of decomposition.

Blubber samples from ESG001 were used in the body location portion of this study. Duplicate full depth blubber samples were collected from all six sites shown in Fig. 1. These samples were wrapped in aluminum foil, placed in Teflon bags and stored at −80 °C. Full depth blubber was sampled from the mid-thoracic region just below the caudal insertion of the dorsal fin (Fig. 1, site 3) of the remaining five individuals in Table 1 for the blubber layer analysis. After initial collection, these samples were placed in freezer bags and stored at −20 °C until subsampling. Samples were partially thawed and subsampled, with subsamples stored at −80 °C or below until analysis.

2.2. Histological Preparation and Analysis

Samples from sites 3, 4 and 5 (Fig. 1) from ESG001 and samples from site 3 of the remaining five animals (Table 1) were used to determine depth-dependency of POPs and FAs. Prior to sectioning, a portion of each sample was histologically prepared, embedded in paraffin, stained with hematoxylin and eosin (H&E) stain and mounted according to Schmacher et al. (1993). The slides were placed on a light box and digital photographs were taken using a VideoLabs Student Cam™ (Golden Valley, MN, USA) ocular camera. Images were then imported into an image editing program, analySIS® (Soft Imaging System GmbH,

Table 1
Level A data from six subadult male bottlenose dolphins that either stranded or were incidentally killed on the coasts of North Carolina and Virginia between 1999 and 2006. Animal body masses that could not be obtained are listed as "NE". SI (Smithsonian Institute) Code indicates the degree of decomposition of the carcass. Code 2 is a fresh dead animal with no skin peeling and organs intact.

Animal ID	Length (cm)	Mass (kg)	Body condition	Reproductive status	Sample collection date	Strand location	SI code
EMM 006	197	NE	Robust	Immature	6, May 1999	Surf City/North Topsail, NC	2
PTM 117	189	73.6	Not emaciated	Immature	15, March 2000	Emerald Isle, NC	2
SAE 003	173	NE	Robust	Immature	20, April 2002	Wrightsville Beach, NC	2
VMSM 2000-1049	207	114.0	Robust	Immature	29, September 2000	Norfolk, VA	2
WAM 572	196	93.0	Robust	Immature	10, November 2002	Sunset Beach, NC	2
ESG 001	203	114.4	Not emaciated	Immature	31, October 2006	Atlantic Beach, NC	2

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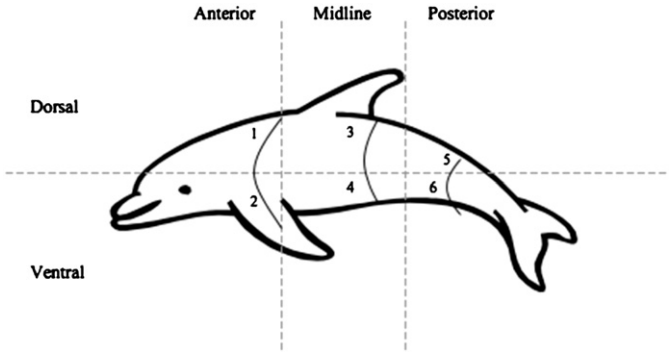

Fig. 1. Bottlenose dolphin blubber sampling sites. Site 1: 10 cm cranial to the leading edge of the dorsal fin and 10 cm down from the dorsal midline. Site 2: 10 cm cranial to the leading edge of the dorsal fin and 10 cm up from the ventral midline. Site 3: Biopsy site. Site 4: 10 cm up from ventral midline, below the caudal insertion of the dorsal fin (below biopsy site). Site 5: Even with the anus, 10 cm down from the dorsal midline. Site 6: Even with the anus, 10 cm up from the ventral midline.

Lakewood, CO). A horizontal axis was drawn across the blubber image and the percent green hue saturation of each pixel along that axis was calculated using the software (Fig. S1). Green saturation values were used as a proxy for structural fiber distribution; lower saturation values equated to greater structural fiber densities. Saturation values consistently one integer above the average green saturation value along the horizontal axis for a distance of at least 0.5 mm were considered part of the "middle" layer, while any saturation values below this average (for at least a distance of 0.5 mm) were considered part of the "deep" layer if located on the muscle side, or "superficial" layer if located on the skin side. Measurements were then taken from the skin to the shallowest edge (side closest to the skin) of each of these layers to determine the depth of each layer. Three replicate measurements were made for each image to ensure consistency (<10% difference between measurements) and the average depth of each layer was calculated and applied to the remaining full depth sample to determine where slices would be made.

2.3. Sample Slicing, Extraction, Cleanup

Each depth-dependent sample was stored at −20 °C for at least 10 min prior to slicing to allow for successful slicing with minimal lipid loss. Skin, muscle and exposed edges were trimmed and full depth blubber was sliced into sections using a Bron stainless steel mandoline (Bron Coucke S.A., Orcier, France). The mandoline was sonicated, scrubbed, rinsed with deionized water, and rinsed with acetone followed by hexane three times before each cut was made. After the blade was adjusted to the correct height, a slice was made and the sub-sample was measured to the nearest mm using a ruler to ensure the cut was of the desired depth. Excess tissue was trimmed. In addition to the layer samples, full depth sections of each depth-dependent sample and full depth samples pertaining to the body location analysis were analyzed as well.

Samples were analyzed for POPs as described elsewhere (Litz et al., 2007). Briefly, approximately 0.4 g to 1.4 g of blubber was macerated and then extracted with dichloromethane by pressurized fluid extraction (Dionex, Salt Lake City, UT, USA) following the addition of an internal standard solution consisting of 26 isotopically labeled polychlorinated biphenyl (PCB) congeners, polybrominated diphenyl ether (PBDE) congeners and organochlorine pesticides. Sample extracts were evaporated to 0.5 mL or the lowest volume possible in a stream of purified N_2 (TurboVap II, Biotage, Uppsala, Sweden) and lipid content (as total extractable organics) was determined gravimetrically from a subsample of the extract. Depending on the remaining volume, from 0.1 g to 1 g of the sample extract was removed and stored at −20 °C to be used for FA determination. For the remainder of the extract (approximately 50% by volume), high molecular mass compounds were removed by size exclusion chromatography and extracts were further cleaned up prior to POP analysis by solid phase extraction using alumina (Litz et al., 2007).

2.4. POP Measurement

A total of ninety-five PCBs, PBDEs and pesticides were determined using gas chromatography/mass spectrometry (GC/MS; Agilent 6890/5973, Wilmington, DE, USA) operated in the electron impact (EI) mode using the column and instrumental conditions given elsewhere (Litz et al., 2007). Samples were also analyzed for 15 organochlorine pesticides by GC/MS in the negative chemical ionization (NCI) mode as described elsewhere (Litz et al., 2007). POP concentrations were determined from a six point calibration curve prepared from Standard Reference Materials (NIST SRM 2261 Chlorinated Pesticides in Hexane, SRM 2262 Chlorinated Biphenyl Congeners in Isooctane, SRM 2274 PCB Congener Solution-II in Isooctane, SRM 2275 Chlorinated Pesticide Solution-II in Isooctane) and additional solutions. For quality control, one blank and one aliquot of SRM 1945 Organics in Whale Blubber were also run along with each batch of blubber samples. Results agreed to within 20% of certified values for SRM 1945 and overall analytical variability based on repeated analyses of SRM 1945 ($n = 3$) was 9.4%.

2.5. Fatty Acid Esterification and Analysis

FAs were converted to butyl esters as described previously (Koopman et al., 1996). Fatty acid samples were analyzed by GC-flame ionization detection (GC-FID; Varian 3800 Autosystem GC, Walnut Creek, CA, USA) using either a 30 m × 0.25 mm i.d. capillary column coated with 50% cyanopropyl polysiloxane (0.25 μm, DB-23; J&W Scientific, Folsom, CA) or a 30 m × 0.25 mm i.d. column coated with nitroterephthalic acid modified polyethylene glycol (0.25 μm, ZB-FFAP; Phenomenex, Torrance, CA) using temperature programs described previously (Zahorodny Duggan et al., 2009). FAs were identified by comparison of retention times with those from NuChek Prep Standard Solution 463 (Nu Chek Preparations Inc., Elysian, MN, USA). GC response factors for each FA were used to calculate weight percentages and were based on modified theoretical response factors (Perkins, 1991) that were adjusted according to values from known standard mixtures (Iverson, 1988). Identified FAs were converted to mass fraction (%) relative to the total mass of FAs in the sample extract.

2.6. Statistical Analyses

Statistical analyses were conducted using the freeware software package R (version 2.4.0; The R Foundation for Statistical Computing, Chapel Hill, NC). Shapiro–Wilks tests were used to assess data distribution and log transformations were applied to non-normal data. Bartlett tests were used to monitor homogeneity of variance. Because the number of response variables was far greater than the number of samples analyzed, similar contaminant classes (ΣPCB, ΣDDT, ΣHCH and ΣPBDE) and similar lipid types (dietary and biosynthesized) were grouped and summed before analysis (Table S2). For additional analyses, PCB compounds were also grouped by both homolog and metabolic groups (Yordy et al., 2010b).

For the blubber depth analysis, linear mixed-effects models (LMEs) were used to compare wet mass contaminant concentrations and lipid-normalized contaminant concentrations among full depth, superficial, middle and deep layers. FA distribution and percent lipid among blubber layers were analyzed using LMEs and LMEs were also used to find correlations between percent lipid and hue saturation values obtained from the histological analysis. To determine significance, a Sequential Bonferroni correction was applied to the results in order to take into account multiple comparisons being made between layers and minor deviations from normality. Two-way analyses of variance

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(ANOVAs) were used to compare wet mass and lipid-normalized concentrations of contaminants and percent lipid and FA distributions among body locations. Two-way ANOVAs were also used to make the same comparisons among layers and full depth samples at various body locations, with a significance level of $p < 0.05$. Tukey's Honest Significant Difference tests were used to determine where differences between layers and locations occurred. Non-parametric two-way ANOVAs on ranks were used on data that were non-normal and Bonferroni corrections were applied to the data to determine where significant differences between layers and locations occurred. Results are reported as *p*-values in all cases.

3. Results and Discussion

3.1. Histological Results

All results for the depth-dependent analyses were based on six individuals. Since hue saturation was used as a proxy for structural fiber and by extension adipocyte distribution, it was important to test for a correlation between lipid distribution and hue saturation values. Lipid content and adipocyte size are known to increase in the middle and deep layers of bottlenose dolphin blubber with age (Struntz et al., 2004). Although adipocytes in the deep blubber layer shrink during weaning, adipocyte size in the superficial blubber varies little with nutritional status (Struntz et al., 2004), suggesting the superficial blubber layer serves a more structural role. Lipid concentrations were expected to be high in areas with high hue saturation values, i.e. low structural fiber densities and larger adipocytes. There was a significant correlation ($R^2 = 0.78$, $p < 0.001$) between lipid concentration and hue saturation values in sectioned blubber, confirming that the separation technique distinguished between physiologically different blubber layers. Although others have separated the blubber of various species into layers, (Aguilar and Borrell, 1991; Evans et al., 2004; Koopman et al., 1996; Tilbury et al., 1997), this is the first quantitative technique that has been used to separate blubber into physiologically different segments based on structural characteristics. This method could be extended to other marine mammals species provided the technique is validated with respect to lipid or adipocyte distribution.

3.2. POP and FA Variability with Body Location in a Single Individual

There were no significant differences in FA distribution with horizontal body location (Tables 2 and 3) as was found in a prior study of bottlenose dolphin blubber (Samuel and Worthy, 2004). This finding differs from harbor porpoises, in which blubber sampled from the caudal peduncle contained different proportions of some FAs (i.e. iso:5:0, 16:1n-7, 22:1n-11) than was found at other body sites, which may relate to the locomotor function of blubber in the peduncle region (Koopman et al., 1996).

Wet mass summed POP concentrations were significantly different among body locations on a single animal (Tables 2 and 3). Σ_{81}PCBs, Σ_6DDTs and Σ_7PBDEs were significantly different between anterior and posterior regions and ΣPCBs were also different between anterior and midline regions, with anterior regions having the highest concentrations (18,700 ng/g wet mass to 22,400 ng/g wet mass). Concentrations in the posterior region were expected to be lower than in the anterior and middle region because the tailstock blubber is thought to be less metabolically active than the thoracic blubber (Pabst, 1996); however, this pattern was not observed for all contaminant groups.

These results suggest that contaminant distribution is heterogeneous with bottlenose dolphin body location. POPs are highly lipophilic and partition into the lipid phase, so it is possible that POP variability with body location was a function of the lipid distribution across the body (Aguilar, 1985). Blubber lipid content differed among body locations and differed significantly among dorsal and ventral sites (Tables 2 and 3). When wet mass POP concentrations were lipid-normalized the number of significant differences among body location comparisons declined from 12 to six indicating that differences in body location on a wet mass basis were partly driven by blubber lipid differences. Similarity of lipid-normalized POP concentrations across body locations has been observed in striped dolphin, *Stenella coeruleoalba*, (Tanabe et al., 1981) and harbor porpoise, *Phocoena phocoena* (Tilbury et al., 1997). The most consistent difference regarding lipid-normalized POPs was seen with body location, where significantly higher POP concentrations were found in ventral than in dorsal blubber.

There was evidence of slight preferential partitioning of recalcitrant POPs into ventral versus dorsal blubber. For example, recalcitrant PCB congeners (those with no adjacent hydrogen atoms on the biphenyl ring) were slightly higher in anterior ventral versus anterior dorsal blubber (Fig. S2). In addition, dietary and biosynthesized FAs were significantly different between dorsal and ventral sites, with higher dietary FAs occurring in the dorsal blubber and higher biosynthesized FAs in the ventral blubber (Table S1). This suggests that the dorsal blubber may be more metabolically active than the ventral blubber in this individual and hence dorsal blubber may reflect POPs obtained from more recent feeding events.

3.3. POP and Fatty Acid Variability with Body Location and Depth in a Single Individual

FA distribution with blubber depth varied among three different body locations of a single individual (Table S1). Significantly more dietary FAs ($n = 20$) were present in the deep and middle blubber layer than the superficial layer and significantly more biosynthesized FAs ($n = 12$) were present in the superficial layer than the middle and deep layers (Table 3), again supporting the assumption that superficial blubber assumes a greater structural role than deep blubber. These results are in agreement with other studies from various species (fin whale, *Balaenoptera physalus*; (Lockyer et al., 1984) harbor porpoise; (Koopman et al., 1996) bottlenose dolphin; (Samuel and Worthy, 2004)) and suggest FAs obtained from food items are generally deposited in the metabolically active deep blubber layer, while endogenous FAs are generally found in the superficial blubber layer. This may be due to greater microvascularity in middle and deep blubber layers compared to the superficial layer (McClelland et al., 2012).

Because blubber was taken from three different body locations for the depth-dependent analysis, this variable was included in the statistical model used to compare POP concentrations (Table 3). Three compound classes were significantly different among the three locations on a wet mass basis; however POP classes were not significantly different on a lipid-normalized basis (Table 3). All contaminant groups were significantly different among deep and superficial, and deep and middle layers, however percent lipid only differed between deep and middle layers (Table 3). Differences in POP concentrations between the deep and middle layer may be explained by the lipid distribution, as lipid-normalized concentrations were either not significant or much less significant than their wet-weight counterparts. Because the middle layer of blubber is comprised of larger adipocytes and fewer structural fibers (Struntz et al., 2004), percent lipid was significantly greater in the middle layer when compared to the superficial and deep layers, though percent lipid was not significantly different between the deep and superficial layers. When the POP data were lipid-normalized, the superficial layer still contained significantly greater concentrations of ΣPCBs, ΣDDTs, ΣPBDEs, and ΣChlordanes than the deep layer. The average difference between deep and superficial layers at all body locations was 68.6%, 59.6%, 64.1% and 20.0% for ΣPCBs, ΣDDTs, ΣPBDEs and ΣChlordanes, respectively. These differences were reduced to 55.8%, 46.6% and 51.1% for ΣPCBs, ΣDDTs and ΣPBDEs, respectively, and increased to 39.7% for ΣChlordanes after lipid normalization. ΣHCH concentrations were significantly different between the middle and superficial layers on a wet mass basis, as was percent

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Table 2
Summary of POP concentrations and FA distribution among blubber depths in subadult male bottlenose dolphins. POP are in ng/g wet mass, lipid-normalized contaminant concentrations are in ng/g lipid, percent lipid is in percent and dietary and biosynthesized FA values are in mass fraction (%). Significant differences at the $\alpha = 0.05$ level were detected using linear mixed-effects models and a Bonferroni correction was applied to the results. "NS" indicates non-significant results.

Individual	Layer	ΣPCBs		ΣDDTs		ΣHCHs		ΣPBDEs		ΣChlordanes		% Lipid content	Dietary fatty Acids	Biosynthesized fatty acids
		Wet mass	Lipid norm	Wet mass	Lipid norm	Wet mass	Lipid norm	Wet mass	Lipid norm	Wet mass	Lipid norm			
EMM 006	Full	35,300	62,000	11,200	19700	24.6	43.2	2380	2380	2240	3930	57.0		
	Deep	42,400	96,300	12,300	28800	22.1	10.2	2720	3720	2510	1160	42.6	22.5	14.5
	Middle	42,200	61,100	13,600	19600	28.1	40.7	2850	2850	2750	3990	69.0	15.5	17.6
	Superficial	19,600	65,200	5980	20700	14.1	15.4	1280	1840	1260	1380	28.9	8.25	22.6
PTM 117	Full	12,700	18,600	3960	5770	20.4	29.8	840	837	765	1120	68.5		
	Deep	23,100	32,900	5740	8460	20.6	22.0	1310	2190	1180	1250	67.8	11.9	15.0
	Middle	14,000	18,000	4530	5810	24.1	30.8	960	958	900	1150	78.1	13.5	17.6
	Superficial	5450	15,500	1600	4810	11.4	16.6	355	651	334	484	33.3	11.5	19.6
SAE 003	Full	15,400	24,100	6180	9700	19.0	29.9	1340	1340	1130	1770	63.7		
	Deep	17,100	24,100	6100	8880	19.2	14.5	1890	728	1190	897	68.6	13.1	12.9
	Middle	18,000	23,200	7440	9550	23.8	30.5	1580	1580	1390	1780	77.9	9.45	17.9
	Superficial	17,700	44,7000	6290	164,000	12.6	46.4	1760	1050	1280	4720	3.8	7.67	18.8
VMSM 2000-1049	Full	19,800	52,600	7900	20,900	14.4	38.1	1230	1210	972	2580	37.7		
	Deep	7420	41,400	3000	16,800	2.97	12.5	335	895	385	1580	18.0	17.7	13.2
	Middle	26,000	48,400	10,400	19,300	19.2	35.6	1310	1670	1350	2510	54.1	14.9	15.4
	Superficial	8960	60,400	3780	25,400	3.29	20.3	480	1490	464	2770	14.8	12.6	17.5
WAM 572	Full	20,200	58,400	6110	17,700	8.88	25.6	1240	1230	1120	3240	34.6		
	Deep	37,900	71,8000	10,600	207,000	8.15	8.09	2140	1390	2040	2020	5.1	20.6	12.9
	Middle	30,200	53,100	8980	15,800	8.86	15.6	1350	1350	1700	2990	56.8	18.4	15.2
	Superficial	5480	77,400	1290	18,600	1.15	16.6	229	953	256	3690	6.9	12.0	15.7
ESG 001	Full	15,900	25,700	5330	8610	13.4	6.46	775	1250	89.1	1290	61.9		
	Deep	7710	19,200	3000	7360	9.63	11.6	396	986	85.4	1160	40.2	19.9	10.5
	Middle	16,700	23,000	5580	7720	16.2	9.26	806	1120	105	1120	72.3	18.7	11.9
	Superficial	15,100	35,800	5060	12,000	11.3	12.6	692	1650	113	1830	42.1	11.4	16.4
Significance (df = 10)	Deep vs middle	NS	NS	NS	NS	0.009	NS	NS	NS	NS	NS	0.007	NS	0.002
	Deep vs superficial	NS	NS	NS	NS	0.03	NS	NS	NS	NS	NS	0.04	0.0006	<0.0001
	Middle vs superficial	NS	NS	0.02	NS	0.0002	NS	NS	NS	NS	NS	0.0002	0.01	0.004

lipid. When the data were lipid-normalized, not only did ΣHCH concentrations remain significantly different between the middle and superficial layers, but all other contaminant groups were also significantly different between these two layers. These differences may be explained by the dynamic relationship that exists between blubber and blood with respect to lipid metabolism and POP mobilization. A negative correlation has been found between blubber lipid content and blood plasma POP concentrations, suggesting that when lipids are being metabolized, such as in times of nutritional stress, POPs are mobilized from the blubber to the blood compartment (Yordy et al., 2010c). Because the middle and deep blubber layers are more metabolically active than the superficial layer, the majority of lipids and contaminants are most likely coming from these deeper layers, while contaminant concentrations in the superficial layer remain more constant. This pattern would create a POP concentration differential between the metabolically active and inactive blubber layers.

Individual ESG001, the dolphin used for the body site comparison, was the largest (by mass) subadult animal sampled in this study. Based on size, this individual likely relied more on fish and the shift in diet from milk to fish resulted in the high degree of POP stratification observed between blubber layers and body locations. Since milk generally contains more bioaccumulated contaminants than fish (Yordy et al., 2010b), it is possible that the lower contaminant levels from the fish were being deposited in the more metabolically active deep blubber layers, while the contaminants from the milk that have been accumulating throughout the lifetime of the animal were sequestered in the less metabolically active superficial layers (Ridgway and Reddy, 1995). This may in fact be the case in subadult animals as adipocytes in the deep blubber layer are smaller than those found in the middle layer (Struntz et al., 2004) suggesting that deep blubber layer lipids are labile and both lipids and contaminants that are found there represent recent feeding events as opposed to long-term accumulation. Although some work has been done on the FA composition of fish present in the diet of bottlenose dolphins off the coast of South Carolina (Recks, 2004), there have been no studies of FA composition of bottlenose dolphin milk compared to prey fish. These data would be invaluable to help determine the process by which stratification of POPs occurs in the blubber of subadult bottlenose dolphins.

The distribution of individual POPs between deep and superficial blubber is shown in Fig. S3. A 1:1 relationship with all compounds falling on the same line would indicate similar partitioning between deep and superficial blubber. This pattern was not observed and instead recalcitrant PCBs were found in greater proportions in the superficial layer. For example, PCBs 153, 180, 187 and 194 lay above the 1:1 line indicating preferential accumulation in superficial blubber. This difference was significant when summed concentrations of major recalcitrant PCBs (132 + 153, 146, 172, 175, 178, 180, 183, 187, 191, 194, 195, 196 + 203, 201, 202, 205, 206, 207, 208 and 209) and total concentrations were compared between deep and superficial layers using a two-way ANOVA ($p = 0.0003$). The retention of higher molecular weight compounds likely stems from the greater mobility of lower molecular weight compounds relative to higher molecular weight compounds (Yordy et al., 2010c).

3.4. POP and FA Variability with Depth in Six Individuals

FA distribution with blubber depth in multiple individuals was heterogeneous, with significantly higher concentrations of dietary FAs in the deep and middle layers compared to the superficial layer (Table 2). In addition, biosynthesized FAs were significantly different among all layers with concentrations of biosynthesized FAs increasing from the deep to superficial layer. Similar results were found in other studies from various species (Koopman et al., 1996; Lockyer et al., 1984; Samuel and Worthy, 2004; Wells et al., 2005). The distribution of FA with body location (dorsal vs. ventral) and blubber depth was consistent and this pattern was also observed in white and killer whales (Krahn et al., 2004) suggesting that the metabolic activity of blubber varies with blubber depth and location and that this pattern may be phylogenetically conserved among odontocetes.

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Table 3
Statistical results (p values) from comparisons made between POP concentrations and FAs from various body locations and blubber depths of a single subadult male bottlenose dolphin. Significant differences ($p < 0.05$) were detected using a 2-way analysis of variance. Tukey's Honest Significant Difference (HSD) tests were used to determine significant differences among individual layers or locations and a Bonferroni correction was applied to the results. "NS" indicates non-significant results. Superscripted letters correspond to the first letter of the location or layer with the higher concentration in the comparison. "*" indicates a significant difference was found using the ANOVA, but there were no significant differences among specific layers or locations using the Tukey's HSD test.

Location	Location	ΣPCBs		ΣDDTs		ΣHCHs		ΣPBDEs		ΣChlordanes		% Lipid Content	Dietary Fatty Acids	Biosynthesized Fatty Acids
		Wet Mass	Lipid Norm	Wet Mass	Lipid Norm	Wet Mass	Lipid Norm	Wet Mass	Lipid Norm	Wet Mass	Lipid Norm			
Horizontal (df = 2) and vertical (df = 1) location results	Anterior vs midline	0.02 ^{A}	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS
	Anterior vs posterior	0.009 ^{A}	NS	0.03 ^{A}	NS	NS	*	0.02 ^{A}	NS	NS	NS	NS	NS	NS
	Midline vs posterior	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS
	Dorsal vs ventral	NS	NS	NS	0.01 ^{V}	NS	NS	0.05 ^{V}	0.001 ^{V}	NS	0.02 ^{V}	0.02 ^{V}	0.006 ^{D}	0.02 ^{V}
Body location (df = 2) with depth (df = 2) results	Middorsal vs midventral	NS	NS	NS	NS	*	NS	*	NS	NS	NS	NS	NS	NS
	Middorsal vs postdorsal	NS	NS	NS	NS	*	NS	*	NS	NS	NS	NS	NS	NS
	Midventral vs postdorsal	NS	NS	0.05 ^{MV}	NS	*	NS	*	NS	NS	NS	0.03 ^{MV}	NS	NS
	Deep vs middle	<0.0001 ^{M}	0.04 ^{M}	<0.0001 ^{M}	NS	0.0003 ^{M}	NS	<0.0001 ^{M}	0.02 ^{M}	NS	NS	<0.0001 ^{M}	NS	NS
	Deep vs superficial	<0.0001 ^{O}	0.003 ^{O}	<0.0001 ^{O}	0.003 ^{O}	0.003 ^{O}	NS	<0.0001 ^{O}	<0.0001 ^{O}	NS	0.003 ^{O}	NS	<0.0001 ^{I}	<0.0001 ^{O}
	Middle vs superficial	NS	0.002 ^{O}	NS	0.002 ^{O}	0.003 ^{O}	0.001 ^{O}	NS	<0.0001 ^{O}	NS	0.0005 ^{O}	0.0001 ^{M}	<0.0001 ^{M}	<0.0001 ^{O}

To assess POP stratification with blubber depth in multiple subadult individuals, contaminant group concentrations in full depth blubber were first compared to deep, middle and superficial blubber layers. Wet mass ΣHCH concentrations were significantly higher in full depth samples compared to superficial blubber ($p = 0.0003$). When data were lipid-normalized, ΣHCH concentrations were significantly greater in full depth samples when compared to deep blubber layer samples ($p = 0.009$). No other contaminant groups were significantly different. These results suggest that statistically similar concentrations could be calculated for the majority of contaminants whether a partial depth biopsy or a full depth sample was used. However, to assess variation in contaminant concentration on a finer scale, the full depth data were removed from further analyses and layers were compared.

Wet mass ΣHCH concentrations were significantly different among all blubber layers compared and ΣDDT concentrations were significantly different between middle and superficial layers (Table 2). However, percent lipid also varied significantly among blubber layers. When POP data were lipid-normalized, no contaminant groups were significantly different among layers. Therefore, differential patterns in contaminant storage among blubber layers are partly a function of lipid distribution. Tilbury et al. (1997) found lipid content was not significantly different among three sections of harbor porpoise blubber (n = 2), however wet mass ΣPCB concentrations were significantly different between deep and superficial and middle and deep layers. Although the results obtained from harbor porpoise blubber conflict with those obtained here, the former should be carefully considered as the statistical tests were not performed on lipid-normalized data. Therefore, heterogeneous POP distribution in harbor porpoises could be due solely to lipid distribution.

Although POP groups were not significantly different with blubber depth, the variability of homolog group distribution with depth was also assessed. Wet mass homolog group concentrations in the deep layer were plotted against wet mass homolog group concentrations in the superficial layer, with all data represented as a proportion of ΣPCBs found in that layer (Fig. 2). All homolog groups were evenly distributed around a 1:1 line, suggesting that homolog group distribution is also homogenous with blubber depth. The three layers were compared, two at a time, in this same way revealing similar results.

Contaminant distribution was homogenous with blubber depth when data from these six individuals were analyzed; however it is important to note that in both the present study and Tilbury et al. (1997), the patterns observed with blubber depth were not consistent among individuals. Here, lipid-normalized ΣPCBs were highest in the deep blubber layer of EMM006, but in VMSM 2000-1049 lipid-normalized ΣPCBs were highest in the superficial layer (Table 2). The difference in POP stratification among individuals highlights the dynamic nature of this tissue. Yordy et al. (2010c) found that POP partitioning between blubber and blood in bottlenose dolphins was generally related to lipid content in blood. However, depending on nutritional status (blubber lipid content was used as a proxy) blood/blubber portioning was compound class dependent. This observation coupled with a diet history that is highly variable with respect to POP concentrations (i.e., milk vs. fish) apparently leads to high variability in individual POP sequestration in blubber with depth. All of the dolphins in this study were immature and likely in different stages of being weaned, meaning that the proportions of fish and milk in the diet were dissimilar.

To further assess variability between individuals, wet mass contaminant concentrations in the deep layer were compared to wet mass contaminant concentrations in the superficial layer, with all data represented as a proportion of total contaminants found in that layer (Fig. 3). In general, there is little preferential stratification of POPs between deep and superficial blubber. However, VMSM 2000-1049 and ESG001 do show a slight enrichment of recalcitrant PCBs in the superficial versus the deep blubber. These individuals were the largest animals sampled in this dataset and both were likely

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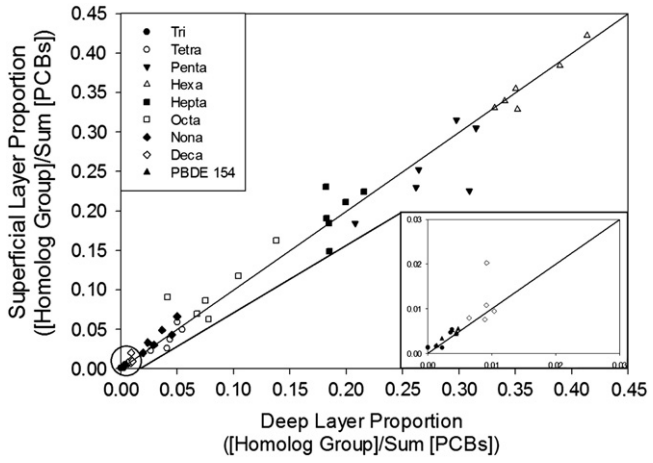

Fig. 2. Fraction of deep layer homolog group concentrations (ng g^{-1} homolog/ΣPCB) plotted against superficial layer homolog group concentrations (ng g^{-1} homolog/ΣPCB) from blubber of six subadult bottlenose dolphins. Points associated with the same homolog group located on the 1:1 line represent those groups which are equally distributed between these two layers.

most reliant on a fish-based diet. Therefore, deposition of POPs from the lower contaminated fish in the metabolically active deep blubber layer may have resulted in the stratification seen with blubber depth. The distribution of contaminants with blubber depth in all other individuals examined was much more homogenous, with compounds in the same metabolic group being evenly distributed around the 1:1 line or adhering closely to the 1:1 line (Fig. 3). Since the remaining animals were smaller and potentially younger, their diet may not have included fish by the time of death, preventing POP stratification in the blubber due to diet shift.

The variability observed in POP concentrations with blubber depth among individuals was quite high (ie. %RSD for ΣPCBs, ΣDDT and ΣPBDEs in middle layers was 49%, 47% and 43%, respectively). However, to monitor the health of populations on the whole, the variability within individuals needs to be considered when designing studies to examine between/among population differences. The high degree of variability associated with these types of data only emphasizes the importance of using a large sample size and controlling for age class, sex, geographic location and obtaining full-depth blubber samples. One potential factor that may affect our observations is declining environmental POP

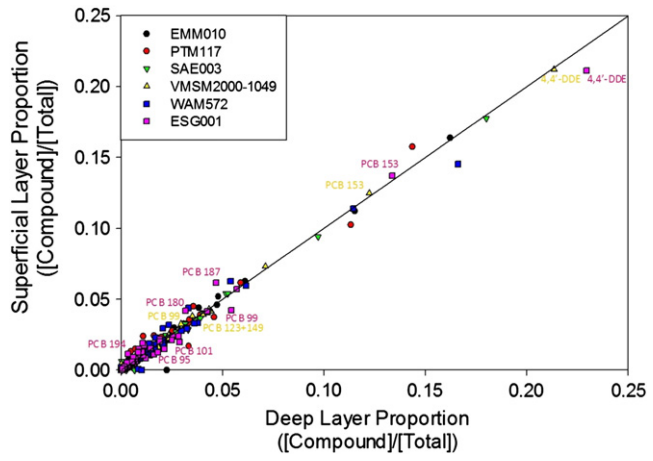

Fig. 3. Fraction of deep layer POP concentrations (ng g^{-1}/total POPs) plotted against superficial layer POP concentrations (ng g^{-1}/total POPs) in blubber from bottlenose dolphins (n = 6). Points located on the 1:1 line represent those compounds which are homogenously distributed among these two layers. Points corresponding to the same metabolic group clustered on one side of the 1:1 line indicate higher concentrations in the layer associated with that side.

concentrations during the span of animal collection. For example, Rigét et al., 2010 reported annual declines of POPs in the Arctic environment on the order of 2% to 3% per year in recent years. Unfortunately, similar temporal trend data are unavailable for marine mammals along the US East Coast. In essence, our statistical comparisons are ratios of concentrations in one blubber layer or location to another hence are concentration independent. Therefore, POPs changing with time in the ambient environment should have negligible effects on the conclusions from our study.

In 2004, Krahn et al. conducted a similar study on fatty acid, percent lipid and POP distribution with blubber depth and body location in white whales (*Delphinapterus leucas*) and killer whales (*Orcinus orca*). Because of its similarities to the present study, a comparison of results is prudent. In both studies, the wet mass of POP classes and individual POPs varied significantly with both blubber body location and with depth, however the variation was reduced when concentrations were based on lipid. Lipid content is therefore an important variable driving POP distribution in blubber. In the present study, lipid-normalized POP classes varied more from dorsal to ventral locations for full depth samples than from anterior to posterior locations on a single dolphin. Lipid-normalized POP concentrations were not significantly different between blubber strata among individual animals however POPs were generally stratified in the blubber of individuals. Krahn et al. (2004) also observed stratification of lipid normalized POPs in white and killer whale blubber, though patterns were not consistent among individuals or species.

4. Conclusions

In bottlenose dolphin, FA concentrations varied systematically with blubber depth. Generally, the presence of dietary FAs decreased from deep to superficial layers and biosynthesized FA concentrations decreased from superficial to deep layers. FAs were also moderately heterogeneous with vertical body location, with higher concentrations of dietary FAs and lower concentrations of biosynthesized FAs found in the dorsal blubber. These patterns are likely due to variability in metabolic activity with blubber depth and body location. Data on capillary profusion patterns in the blubber from different body locations of bottlenose dolphin would be beneficial to further substantiate these results.

The implication to POP data sets derived from remote biopsies where non-full depth samples are collected is that blubber stratification may increase POP variance for the data set but will probably not affect mean lipid-normalized POP concentrations. As both remote biopsy and surgical biopsies are almost always collected from the dorsal side of the animal the anterior to posterior location differences in biopsy location should not affect lipid-normalized POP concentrations in bottlenose dolphin. However, the high degree of variability observed between individuals within a single species provides further support to the need for large sample sizes and controlling for life history variables.

Conflicts of Interest

There are no conflicts of interest among any listed authors.

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or endorsement by the National Institute of Standards and Technology or the National Oceanic and Atmospheric Administration (NOAA), nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2013.06.017.

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