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Anaemia, hypothyroidism and immune suppression associated with polychlorinated biphenyl exposure in bottlenose dolphins (*Tursiops truncatus*)

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Polychlorinated biphenyls (PCBs), persistent chemicals widely used for industrial purposes, have been banned in most parts of the world for decades. Owing to their bioaccumulative nature, PCBs are still found in high concentrations in marine mammals, particularly those that occupy upper trophic positions. While PCB-related health effects have been well-documented in some mammals, studies among dolphins and whales are limited. We conducted health evaluations of bottlenose dolphins (*Tursiops truncatus*) near a site on the Georgia, United States coast heavily contaminated by Aroclor 1268, an uncommon PCB mixture primarily comprised of octa- through deca-chlorobiphenyl congeners. A high proportion (26%) of sampled dolphins suffered anaemia, a finding previously reported from primate laboratory studies using high doses of a more common PCB mixture, Aroclor 1254. In addition, the dolphins showed reduced thyroid hormone levels and total thyroxine, free thyroxine and triiodothyronine negatively correlated with PCB concentration measured in blubber ($p = 0.039$, < 0.001, 0.009, respectively). Similarly, T-lymphocyte proliferation and indices of innate immunity decreased with blubber PCB concentration, suggesting an increased susceptibility to infectious disease. Other persistent contaminants such as DDT which could potentially confound results were similar in the Georgia dolphins when compared with previously sampled reference sites, and therefore probably did not contribute to the observed correlations. Our results clearly demonstrate that dolphins are vulnerable to PCB-related toxic effects, at least partially mediated through the endocrine system. The severity of the effects suggests that the PCB mixture to which the Georgia dolphins were exposed has substantial toxic potential and

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further studies are warranted to elucidate mechanisms and potential impacts on other top-level predators, including humans, who regularly consume fish from the same marine waters.

Keywords: endocrine disruptors; polychlorinated biphenyls; immune suppression; thyroid; marine mammals

1. INTRODUCTION

Polychlorinated biphenyls (PCBs), persistent chemicals widely used for a variety of industrial purposes until banned in the late 1970s, are ubiquitous environmental contaminants associated with a broad range of toxic effects. Experimental as well as epidemiological studies have provided evidence that PCBs can adversely impact immune, endocrine and reproductive systems [1–3]. Exposure to environmental PCBs has been linked to detrimental impacts on wildlife and monitoring of sentinel populations has been proposed to warn of potential human exposure risk as well as to provide insight into potential human health effects [4].

Extreme concentrations, up to 2900 mg kg^{-1} lipid, of PCBs were documented in bottlenose dolphins along the southern coast of Georgia (GA), United States of America (USA) [5,6]. The PCBs exhibit a distinct pattern of highly chlorinated octa- through deca-chlorobiphenyl congeners, characteristic of Aroclor 1268, an uncommon technical PCB mixture. Aroclor 1268 was used at a former chlor-alkali facility, Linden Chemicals and Plastics (LCP) located on the banks of the Turtle/Brunswick River Estuary (TBRE), near Brunswick, GA, USA (figure 1). High concentrations of PCBs characteristic of Aroclor 1268 were also reported in soil, marsh sediments and marine biota sampled in close proximity to the LCP site [7,8]. However, the PCB concentrations reported in bottlenose dolphins, an apex predator in the TBRE, are among the highest ever reported in wildlife [6]. Even more concerning is that the high PCB concentrations are not localized to the TBRE. Dolphins sampled approximately 40 km northeast near the Sapelo Island National Estuarine Research Reserve (NERR; figure 1) were also found to have PCB concentrations at or above the highest levels reported for dolphins near urban centres such as Biscayne Bay, Florida and Charleston, South Carolina [9,10].

Several infectious disease epidemics resulting in large-scale mortalities have occurred in dolphin populations with high PCB and other persistent organic pollutants (POP) levels [11,12], suggesting, but not demonstrating, a causal link between POP exposure, immune function and susceptibility to disease. While suppressed immune function has been well documented in some marine mammals through experimental feeding [13], health impacts in cetacean species have been more difficult to elucidate owing to ethical and logistical considerations. An early study found decreased immune responses associated with blood concentration of PCBs and DDT in a wild dolphin population near Sarasota, Florida, but the sample size was small ($n = 5$) and the study did not control for confounding factors such as age [14]. A more recent and robust study of harbour porpoises (*Phoecoena phocoena*) quantified the increased risk of infectious disease mortality in association with PCB exposure [15]. However, because the study relied on stranded and by-caught (i.e. dead) porpoises, the mechanisms of toxicity associated with the infection and mortality risk could not be determined.

The highly exposed dolphins from the Georgia coast provide an opportunity to elucidate potential toxic mechanisms and effects of chronic PCB exposure in cetaceans. The potential adverse health effects in the dolphins could have further significance for understanding potential human health impacts related to exposure to an uncommon, highly chlorinated Aroclor mixture. This study details the results of a capture-release health assessment of dolphins from the Georgia coast to examine potential detrimental health effects associated with the extreme PCB exposure.

2. METHODS

(a) *Dolphin capture and processing*

Dolphin sampling was conducted over a two week period, 3–14 August 2009. For the first week, operations were based out of Meridian, Georgia; sampling was concentrated in waters near Sapelo Island NERR (figure 1). The base of operations was moved to Brunswick, Georgia for the second week of the fieldwork and sampling was concentrated around the TBRE.

Methods for dolphin capture-release have been previously described [16,17]. Briefly, dolphins were encircled with a net (366 m long, 7 m deep and 22 cm stretch mesh seine net) and then restrained by handlers. Female dolphins greater than or equal to 220 cm were held in the water until an ultrasound could be conducted to assess pregnancy status. Only non-pregnant females and males were brought aboard a specially designed processing boat for physical examination, weighing and morphometric measurement and diagnostic sampling. Pregnant females were not taken on board the boat and instead an abbreviated in-water examination and sampling was conducted. Standardized data collection was conducted as previously described [16,17].

Blood was drawn from ventral fluke vasculature as described in Schwacke *et al.* [16]. Whole blood was kept cool, wrapped and shipped overnight on ice packs to the University of Connecticut for immunological testing. Whole blood for haematology was immediately stored in an onboard cooler until the end of each day when samples were shipped overnight to the Animal Health Diagnostic Laboratory (AHDL) at Cornell University, Ithaca, NY, USA.

For serum biochemistry and hormone analysis, blood samples were allowed to sit at room temperature until clot formation (approx. 30 min) prior to being centrifuged. Serum was then transferred into cryovials and kept cool until the end of each day when biochemistry samples were shipped overnight on ice packs to the AHDL at Cornell University. Serum for hormone analysis was frozen at $-80^{\circ}C$ and shipped to AHDL at the end of the two week field period.

Surgical biopsies of skin and blubber were taken for contaminant analysis as described by Wells *et al.* [18]. A single tooth was extracted under local anaesthesia from some of

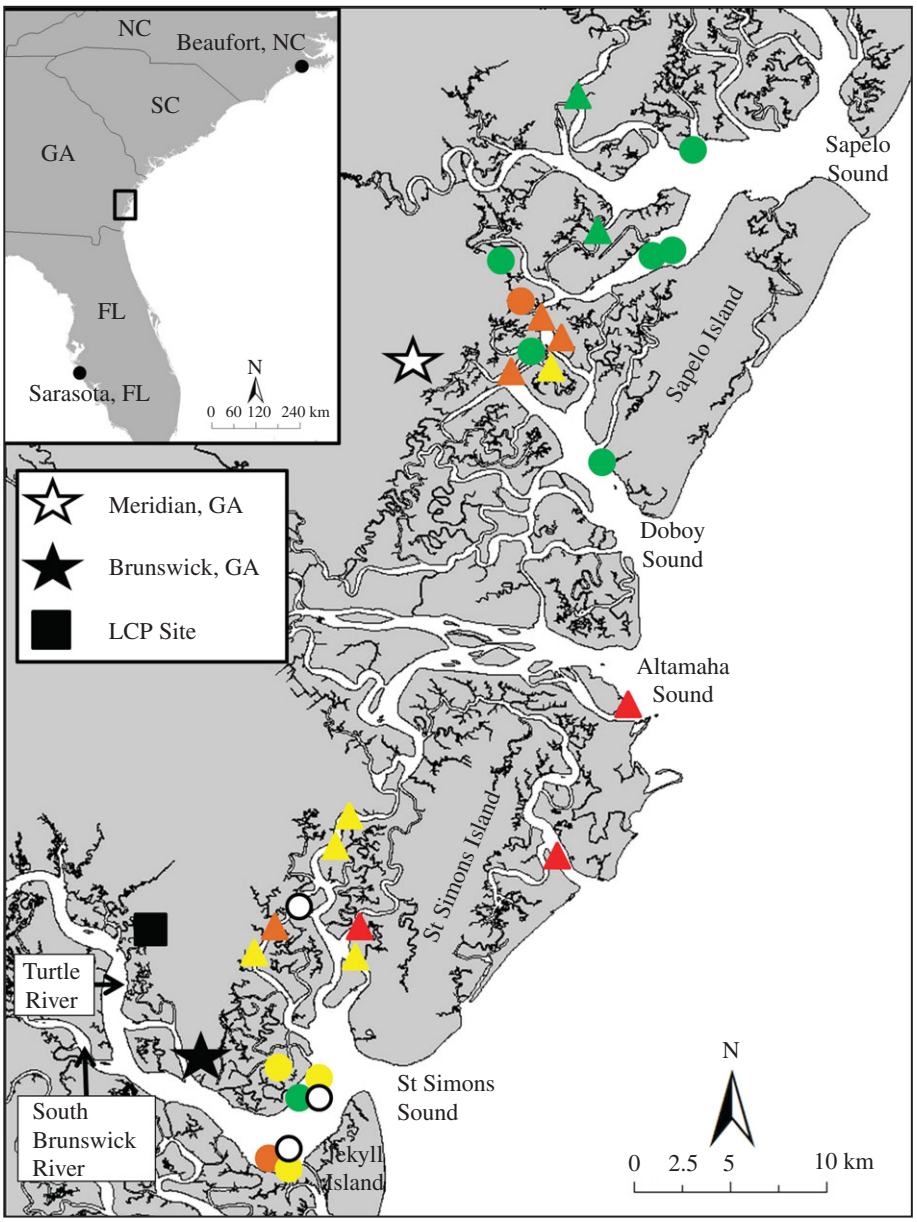

Figure 1. Map of area with dolphin capture locations and PCB concentration ($\mu g\,g^{-1}$ lipid weight) measured in blubber. Circles represent females, triangles represent males. Dolphins sampled in Altamaha Sound and north were categorized as 'Sapelo'; dolphins sampled south of the Altamaha were categorized as 'Brunswick'. PCB $\mu g\,g^{-1}$, white symbols, no data; green symbols, 0–100; yellow symbols, 101–200; orange symbols, 201–300; red symbols, >301.

the dolphins to determine age. Teeth were not taken from dolphins that were apparently very young (less than or equal to 220 cm) or pregnant. In addition, when the lead veterinarian felt that a dolphin was becoming overly stressed, as evidenced by rapid respirations (greater than 8 breaths min^{-1}) or arching, the dolphin was released and a tooth was not taken. Two dolphins were also released early owing to approaching thunderstorms. Teeth were prepared for sectioning using standard procedures [19].

(b) *Laboratory analyses*

(i) *Chemical analysis*

POP including 55 PCB congeners, seven brominated diphenyl ether (BDE) congeners and a suite of organochlorine pesticides were measured in blubber. Methods are described in detail elsewhere [10] and are summarized in the electronic supplementary material.

(ii) *Functional immune assays*

Isolation of leukocytes was performed prior to the evaluation of all immune functions and is described in the electronic supplementary material. Neutrophil and monocyte phagocytosis were evaluated *in vitro* as previously described [20]. Phagocytosis was reported as the percentage of cells that had phagocytized one or more (1+), two or more (2+) and three or more beads (3+). The percentage of cells that phagocytized 3+ beads was used as the endpoint for statistical analysis.

Mitogen-induced lymphocyte proliferation was evaluated *in vitro* as previously described [21]; Concanavalin A (Con A) and lipopolysaccharide (LPS) were used as T-cell and B-cell mitogens, respectively. A stimulation index was calculated as the per cent increase in proliferation in the mitogen-stimulated cells when compared with the unstimulated cells. Further details of immunological assays can be found in the electronic supplementary material.

For quality control purposes, functional immune assays were performed on B6C3F1 mice in parallel with dolphin samples on each day assays were performed. Mouse data were then analysed for outliers to detect and eliminate experiments for which the variability was greater than expected for any technical reason. This quality control programme ensured that technical errors on one given day would not translate in misinterpretation of the data for several dolphins run on that day. Mouse data were also used to facilitate comparisons of samples analysed over time using different reagent batches.

(c) *Data analysis*

Dolphins were categorized into age classes by applying criteria as previously described [16].

(i) *Comparison with baseline values*

Haematological and serum biochemical values were compared with reference intervals for the appropriate age class established from previously sampled wild dolphin populations [22]. Appropriate reference thresholds were not available for endocrine parameters. Therefore, data from two independent dolphin health assessment studies were obtained and used for comparison. Total thyroxine (TT4), free thyroxine (FT4) and total triiodothyronine (TT3) measures were obtained from dolphin health assessment studies near Beaufort, NC, USA ($n = 14$) conducted in April 2006 and Sarasota Bay, FL, USA ($n = 63$), conducted in June 2005, June 2006, May 2008 and May 2009. Protocols for data and sample collection were standardized across the studies, and hormone analysis was performed by the same laboratory at Cornell University. However, the laboratory changed reagents for the measurement of TT3 in August 2006, because the production of the previously used radioimmunoassay was discontinued (see the electronic supplementary material). This may have introduced bias for comparisons of TT3 measurements before and after this date. Inter-assay comparison data collected by the laboratory using reference samples were used to calculate a conversion equation to allow comparison of values analysed using the two different assays. The equation and regression statistics for the inter-assay comparison are given in the electronic supplementary material. The conversion equation was used to adjust dolphin TT3 measurements that were obtained prior to August 2006.

An analysis of variance (ANOVA) was applied to test for differences in TT4, FT4 and adjusted TT3 among sites. Age class was also included as a factor in the model. When a significant difference was determined among sites, a follow-up analysis was conducted to specifically test for differences between the two reference sites and the two Georgia sites (Sarasota and Beaufort versus Sapelo Island and Brunswick) using an *a priori* linear contrast. A critical value $\alpha = 0.05$ was used to assess statistical significance.

(ii) *Relationship of endocrine, immune parameters and PCB concentration*

An analysis of covariance (ANCOVA) was conducted to examine correlation of serum thyroid hormones (TT4, FT4 and TT3) and functional immune indices (Con A stimulation, LPS stimulation, neutrophil phagocytosis and monocyte phagocytosis) with measured blubber PCB concentration. PCBs are lipid-soluble compounds that are readily passed from a lactating female to her nursing offspring [23]. Thus, PCB concentrations measured in adult female dolphins are generally not meaningful indicators of cumulative exposure. For this reason, adult females was excluded from the ANCOVA.

Endocrine data from the reference sites for which concurrent blubber PCB measurements were available were also included in the regression analysis. This included seven and 11 samples from Beaufort and Sarasota, respectively. The seven Beaufort dolphins also had matching functional immune data, which were included in the regression analysis for immune indices. Chemical analysis for the blubber samples from Sarasota and Beaufort was conducted by the same laboratory and the same suite of congeners was summed to derive total PCBs. Age class and sampling site were included as categorical covariates in the ANCOVA; total PCBs (sum of 55 congeners) expressed on a lipid weight basis was included as a continuous covariate.

Data were examined for outlying values and extreme outliers were removed prior to analysis. Details are described in the electronic supplementary material.

Prior to combining the functional immune data from Beaufort collected in 2006 with the data from Brunswick and Sapelo collected in 2009, a one-way ANOVA was performed to compare the mouse control data between the 2 years. We found that mouse proliferation measured in 2006 in conjunction with the Beaufort dolphin samples were significantly higher than the mouse proliferation measures that were made in conjunction with the 2009 Georgia dolphin samples. This is probably owing to slight differences in lots of reagents, kits and supplies across years. Therefore, to avoid the introduction of potential laboratory bias, dolphin data were normalized to the concurrent proliferation measures in mice.

Recent research has indicated a relationship between T-lymphocyte proliferation and TT3 levels [24] so an ANCOVA was also conducted to examine the association of TT3 with the Con A stimulation index.

3. RESULTS

During the 10 day capture-release health assessment, 29 dolphins were captured, examined and sampled (figure 1). The number of dolphins sampled, stratified by site and age-class are provided in the electronic supplementary material. A tooth was obtained from 13 dolphins for age determination, and six females were determined to be pregnant.

Several of the captured dolphins were found to be unusually small for their estimated age. One male whose age was later estimated to be 11 years was only 224 cm. The median length for the adult males (estimated to be at least 10 years of age by dentinal analysis) was only 242 cm. In comparison, the asymptotic length for male dolphins sampled from South Carolina, only 100–500 km to the north of the Georgia study site, is 255.5 cm (95% credible interval = 250.3–261.8) [19]. The median length for adult females from Georgia was 236 cm when compared with 241.5 cm (95% credible interval = 238.5–244.7) reported as the asymptotic length for females from South Carolina [19].

(a) *Haematology and serum chemistry*

Blood samples for haematology and serum chemistry were obtained for 27 and 29 dolphins, respectively. A number of abnormal haematological and serum biochemical parameters were determined for dolphins sampled near both sites (table 1). Most notable, 26 per cent of the sampled dolphins were found to be anaemic, diagnosed as having a haemoglobin value below the 2.5th percentile reference

Table 1. Abnormal haematological and serum biochemical conditions observed in dolphins sampled near Sapelo Island and Brunswick, GA, USA. (Parameter values were compared with reference intervals for appropriate age- and sex-class as described by Schwacke *et al.* [22]. Prevalence calculation based on $n = 27$ samples for haematology (haemoglobin) and $n = 29$ for serum chemistry (all other parameters). For comparison, prevalence was also calculated using samples from Sarasota ($n = 63$) and Beaufort ($n = 12$ and $n = 14$ for haematology and serum chemistry, respectively). GGT, gamma-glutamyl transferase; ALT, alanine aminotransferase; AST, aspartate transaminase.)

condition	defining parameters	no. cases Sapelo	no. cases Brunswick	total cases	prevalence	95% CI	Sarasota prevalence	Beaufort prevalence
anaemia	haemoglobin below lower threshold	3	4	7	0.26	0.11–0.46	0.03	0
elevated hepatic enzymes	one or more above upper threshold: GGT, ALT, AST	4	0	4	0.14	0.04–0.32	0.02	0.07
elevated electrolytes	one or more above upper threshold: sodium, chloride, potassium	1	4	5	0.17	0.06–0.36	0.03	0
elevated lactate dehydrogenase	lactate dehydrogenase above upper threshold	3	6	9	0.31	0.15–0.51	0.10	0.07
hypermagnesemia	magnesium above upper threshold	5	4	9	0.31	0.15–0.51	0	0

threshold. Of the seven anaemia cases, three were normocytic and showed signs of depressed erythropoiesis; two were microcytic concurrent with reduced mean corpuscular haemoglobin, low iron and elevated lactate dehydrogenase; and one was macrocytic with depressed erythropoiesis and indication of mild rouleaux formation with elevation of serum protein. The remaining anaemia case had no other abnormal haematological or biochemical parameters. The three normocytic cases were adult males, and the two microcytic cases were females (one adult and one juvenile, neither pregnant). In addition, two adult males who were within the normal range for haemoglobin demonstrated elevated mean corpuscular volume and mild polychromasia, potentially indicative of recent regenerative anaemia.

Elevated liver enzymes and elevated electrolytes, suggestive of potential hepatic and/or renal pathologies, were noted in 14 and 17 per cent of dolphins, respectively. Elevated LDH, a non-specific enzyme indicative of cell damage, was seen in 31 per cent of dolphins. In addition, hypermagnesemia (magnesium value > 1.7 mEq l^{-1} for adults, >1.8 mEq l^{-1} for calves and juveniles) was found in 31 per cent of the sampled dolphins. The clinical significance of the hypermagnesemia is unclear, but seven of the nine hypermagnesemia dolphins had other indications of health issues, including renal (elevated sodium, potassium, calcium, blood urea nitrogen and/or uric acid) and/or hepatic (elevated alanine amino transferase and/or gamma-glutamyl transferase) concerns.

(b) *Endocrine assessment*

While TT4 did not vary significantly among sites (not shown), TT3 and FT4 concentrations were considerably lower for dolphins sampled in Georgia with the differences most apparent in subadults (figure 2). The ANOVAs supported the conclusion of differences in TT3 and FT4 among sites ($p < 0.001$ for both) and found no difference among sites for TT4 ($p = 0.525$). The age-class covariate term was significant for all three parameters ($p < 0.001$). The linear contrast indicated a significant difference between the two control sites (Sarasota and Beaufort) and the two Georgia sites (Brunswick and Sapelo Island) for FT4 ($p = 0.002$). The p-value for TT3 linear contrast ($p = 0.053$) was only slightly above the established significance threshold.

(c) *Contaminant analysis*

Blubber samples for chemical analysis were stratified into two classes: (i) adult females ($n = 5$), (ii) males and non-adult females ($n = 21$). Measured PCB concentrations ranged from 10.3 to 761 $\mu g\, g^{-1}$ lipid in males and non-adult females and 43.7 to 173 $\mu g\, g^{-1}$ lipid in adult females (figure 1). Further description of results of chemical analysis can be found in the electronic supplementary material.

(d) *Relationship of endocrine, immune parameters and PCB concentration*

Each of the endocrine parameters showed a significant negative relationship with blubber PCB concentration (table 2 and figure 3), although the association between PCBs and TT4 was weaker than for TT3 and FT4. As anticipated, age class also significantly influenced endocrine parameters and was retained in all of the models as a covariate. Site was not indicated as a significant

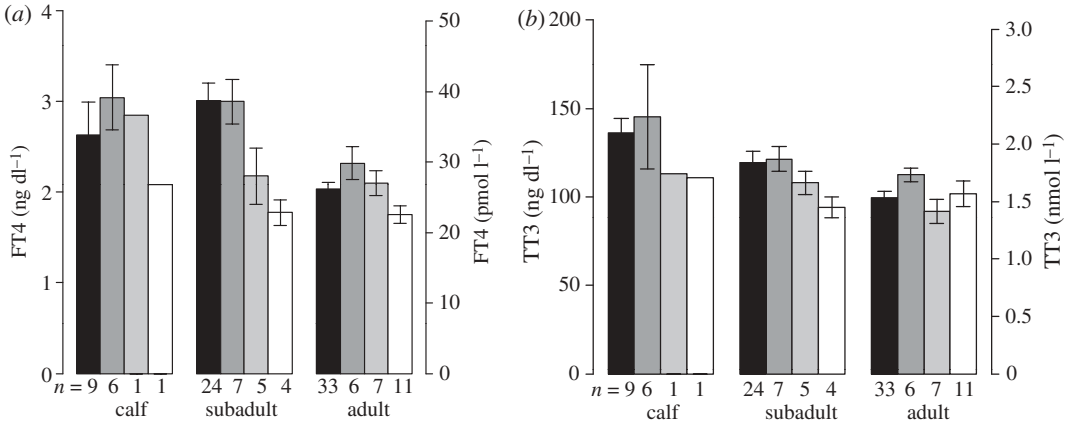

Figure 2. (*a*) FT4 and (*b*) TT3 measured from serum of dolphins sampled near Sarasota, FL; Beaufort, NC; Sapelo Island, GA and Brunswick, GA. Bars represent mean and whiskers represent 95% CI. Sample size (*n*) is indicated below *x*-axis. FT4 differed ($p = 0.002$) between the Georgia sites (Sapelo and Brunswick) and the two reference sites (Sarasota and Beaufort); TT3 was slightly above the critical value ($p = 0.053$). (*a*,*b*) Black bars, Sarasota; dark grey bars, Beaufort; light grey bars, Sapelo; white bars, Brunswick.

Table 2. *p*-values from ANCOVA examining association of serum endocrine values, iron and functional immune indices with natural logarithm of total PCBs measured in blubber. (Model included all males as well as calf, juvenile and subadult females. Length class and site were included as covariate terms. n.s. indicates a non-significant *p*-value. Covariate factors determined to be non-significant were removed from the model and the analysis was repeated to obtain final significance levels.)

	n	model r^2	LN(PCB)	length class	site
endocrine parameters					
TT3	37	0.35	0.009	0.009	n.s.
TT4	38	0.29	0.039	0.012	n.s.
FT4	38	0.79	<0.001	<0.001	<0.001
functional immune parameters					
Con A stimulation index	29	0.56	0.001	n.s.	0.001
LPS stimulation index	31	0.47	n.s.	n.s.	<0.001
neutrophil phagocytosis	30	0.57	0.003	n.s.	<0.001
monocyte phagocytosis	31	0.67	<0.001	n.s.	<0.001
other					
iron	28	0.76	0.018	<0.001	0.01

factor for TT4 and TT3; however, site was retained as a significant covariate for FT4.

Con A stimulation index, an indicator of T-lymphocyte proliferative response, showed a strong positive correlation with TT3 ($p < 0.001$, figure 4) and a negative correlation with PCB concentration (table 2 and figure 4*b*). The two indicators of innate immunity, neutrophil and monocyte phagocytosis, also showed significant negative associations with PCB concentration (table 2). By contrast, LPS stimulation index, an indicator of B-lymphocyte proliferation, did not show a significant association with PCB concentration ($p = 0.377$, table 2).

4. DISCUSSION

Our study clearly demonstrates a multitude of health concerns in dolphins exposed to high concentrations of PCBs, and the observed effects are consistent with adverse effects reported from experimental studies of other mammal species. For example, we found a high prevalence of anaemia in the dolphins sampled along the Georgia coast (table 2), similar to findings in primates after chronic exposure to Aroclor 1254 or 1248 [25,26]. Depressed erythropoiesis with normocytic anaemia was reported for cynomolgus monkeys (*Macaca fascicularis*) after being fed relatively high doses of Aroclor 1254 and both macrocytic and normocytic anaemia were reported after dosing with Aroclor 1248 [26]. We noted similar cases ($n = 3$) of depressed erythropoiesis with normocytic anaemia as well as a macrocytic anaemia in the Georgia dolphins. We also noted two cases of microcytic anaemia with associated iron deficiency. Iron deficiency is often associated with hypothyroidism [27] and the latter two dolphin cases had very low levels of TT3 (0.80 ng ml^{-1} and 0.88 ng ml^{-1}) and FT4 (1.28 ng dl^{-1} and 1.59 ng dl^{-1}). In general, iron levels negatively correlated with blubber PCB concentrations (figure 3).

The PCB concentrations measured from the Georgia dolphins' blubber (maximum = 761 μg g^{-1} lipid) far exceed the concentrations from oil extract of adipose tissue (maximum = 27.5 μg g^{-1}) taken from the laboratory primates at autopsy [26]. The higher concentrations could be in part owing to greater accumulation of the higher chlorinated congeners, which are characteristic of Aroclor 1268. Based on their primate study, Tryphonas *et al.* [26] suggest that the higher chlorinated Aroclor 1254 may accumulate to greater concentration yet have a slightly lower toxic potential when compared with the lesser chlorinated congeners comprising Aroclor 1248. This difference in accumulation and toxicity among PCB mixtures, along with interspecies

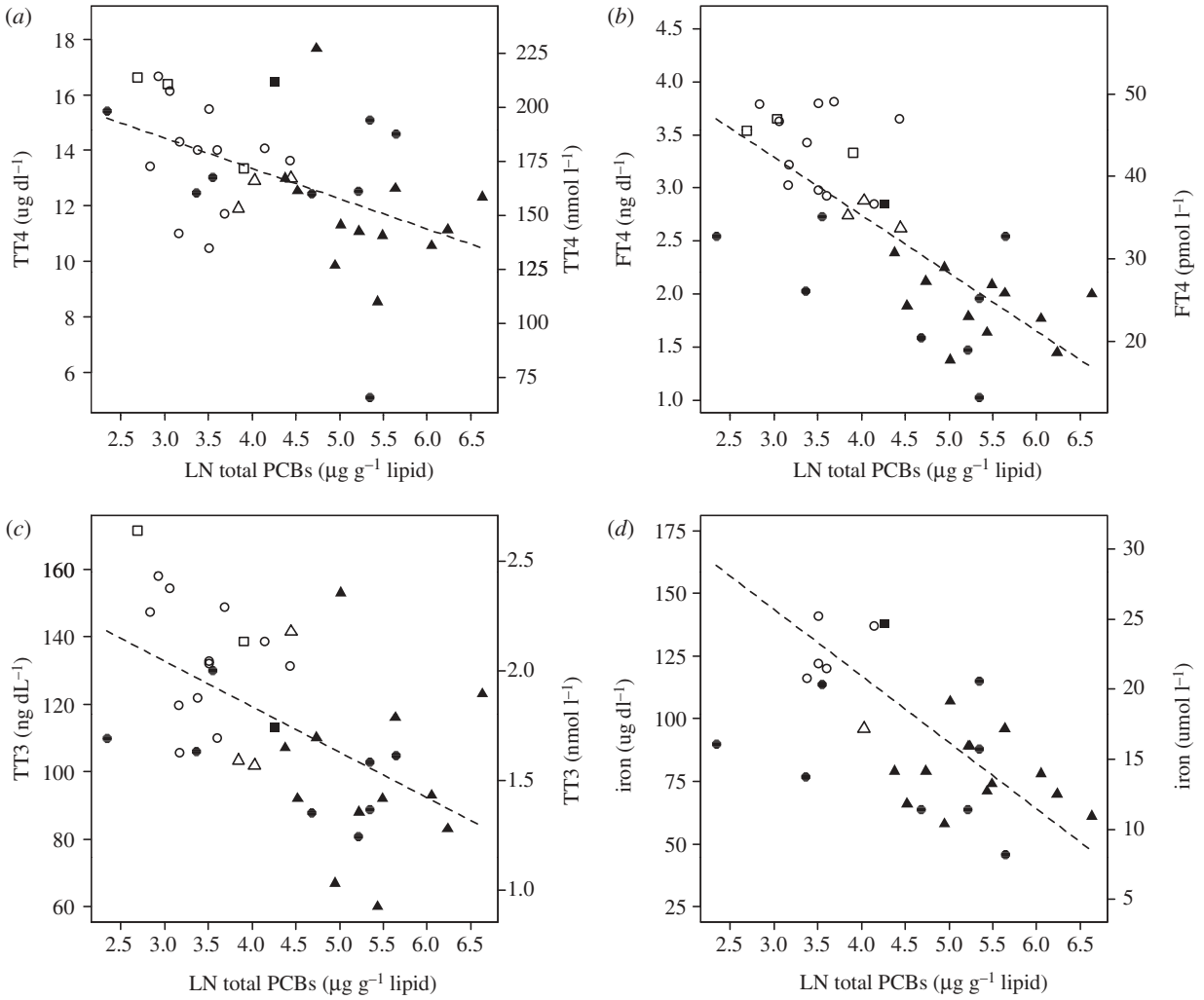

Figure 3. Results from linear model (ANCOVA) examining association of serum (*a*) TT4; (*b*) FT4; (*c*) TT3; and (*d*) iron with natural logarithm (LN) of total PCBs measured in blubber. The model included all males as well as calf and juvenile females sampled near Brunswick, GA, Sapelo Island, GA, Sarasota, FL and Beaufort, NC. Age class and site were included as covariate terms. Circles, squares and triangles represent calves, juveniles and adults, respectively. Solid filled symbols represent samples from the Georgia sites (Sapelo and Brunswick), and hollow symbols represent samples from the reference sites (Sarasota and Beaufort).

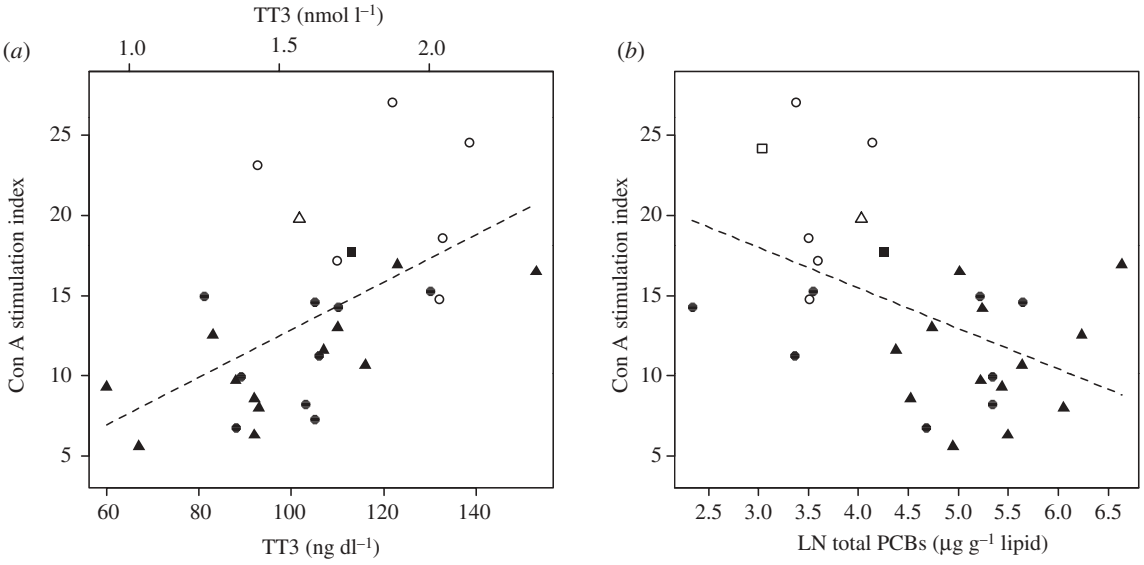

Figure 4. Results from linear model (ANCOVA) examining association of T-lymphocyte stimulation index with (*a*) TT3 and (*b*) natural logarithm (LN) of total PCBs measured in blubber. The model included all males as well as calf and juvenile females sampled near Brunswick, GA, Sapelo Island, GA, Sarasota, FL and Beaufort, NC. Age class and site were included as covariate terms. Circles, squares and triangles represent calves, juveniles and adults, respectively. Solid filled symbols represent samples from the Georgia sites (Sapelo and Brunswick), and hollow symbols represent samples from the reference site (Beaufort).

differences in sensitivity and lipid storage, could explain the dolphins' ability to tolerate the higher PCB tissue concentrations without becoming moribund, which eventually occurred in the laboratory primates after extended dosing.

In addition to the haematological effects, we found severely decreased TT3 and FT4 levels in dolphins sampled from the Georgia coast (figure 2) and the highly significant negative correlation of these hormones with measured blubber PCB concentration (figure 3). This is consistent with the strong evidence of PCB-related thyroid hormone disruption from studies of other marine mammals [28,29] as well as laboratory studies of rats dosed with Aroclor 1254 [30]. The mechanism(s) by which PCBs disrupt thyroid hormones are not completely understood, but experimental evidence suggests that there are potentially multiple toxic pathways. Aside from directly interfering with hormone production in the thyroid gland, at least some PCBs can interact with metabolic enzymes to accelerate clearance of thyroid hormones [2]. Specifically, increased glucuronidation and elimination of thyroxine (T4) through induction of hepatic uridine diphosphate glucuronosyltransferase (UDP-GT) was found in rats fed an Aroclor 1254 mixture [31]. An additional study using specific coplanar PCB congeners suggests that the increased UDP-GT activity could be mediated through the aryl hydrocarbon receptor signal transduction pathway [32]. The PCBs generally associated with this pathway are similar in structure to the dioxins and having one or no chlorine substitutions at the ortho positions on the benzene rings. Although not primary constituents of Arcolor 1268, some of these dioxin-like congeners, particularly PCB 189, were measured in the Georgia dolphins (see the electronic supplementary material).

Alternatively, PCBs could interfere with thyroid hormone transport to peripheral tissues. Some PCBs, as well hydroxylated PCB metabolites, are structurally similar to T4 and may competitively bind to a thyroid hormone transport protein, transthyretin [2]. However, once again the experimental studies to date have examined the role of dioxin-like congeners [33] or the hydroxylated metabolites [34] which are more readily transformed from lower chlorinated PCBs [35]. Thus, competitive binding to transthyretin is not an obvious mechanistic pathway for the primary congeners in the Aroclor 1268 mixture. Furthermore, if there was a high degree of competition for binding to transport proteins, we might expect to see higher levels of unbound T4; we observed lower levels of FT4 in association with PCB concentration.

Regardless of the toxic mechanism, the hypothyroidism observed in the Georgia dolphins could certainly produce a variety of adverse health outcomes as thyroid hormones play a critical role in metabolism and growth. In fact, the dolphins sampled in Georgia were smaller than expected based on measurements of dolphins from other populations. This suggests a potential for reduced growth in the Georgia dolphins, although our sample size (nine males and four females) was too small for meaningful statistical comparisons.

It is unclear whether decreased thyroid hormones also mediated the reduction in innate and acquired immune response. T-lymphocyte mitogen-induced proliferation showed a strong positive correlation with TT3 (figure 4*a*), consistent with a study which found that hypothyroid mice displayed lower T-cell mitogen-induced proliferation but that the administration of triiodothyronine (T3) recovered the proliferative response [24]. The same mouse study provided evidence that T3 has a role in regulating cytokine production which could in turn influence lymphocyte response as well as innate immune response. Although this scenario of thyroid-mediated immune suppression is certainly plausible, we cannot rule out simple covariation owing to the negative association of PCBs with both thyroid and immune endpoints. Innate and adaptive immune functions may also be directly modulated by PCBs. Dolphin T-lymphocyte proliferation and phagocytosis were shown to be significantly reduced upon *in vitro* exposure to simple mixtures of individual PCB congeners [20,21]. Importantly, one of the individual PCB congeners that reduced immune functions upon *in vitro* exposure, PCB 180, is a major component of Aroclor 1268 [7] and was detected in the Georgia dolphin blubber (geometric mean $= 5.1\ \mu g\ g^{-1}$ lipid in males and non-adult females). No matter the mechanism, a lowered T-cell response could increase susceptibility to infectious disease, particularly of viral origin while the suppressed innate immunity could leave animals vulnerable to invasion by bacterial, fungal and protozoan infections. A good correlation was demonstrated between changes in immune function and increased susceptibility to infectious and non-infectious disease in mice in that there were no instances where host resistance was altered without affecting an immune test [36].

While an extensive body of research exists addressing the health effects of PCBs (reviewed in [37]), most studies to date have focused on the widely used PCB mixtures with low to moderate chlorine content such as Aroclors 1248, 1254 and 1260. Less than 1 per cent of the PCBs sold in the USA from 1957 to 1975 were Aroclor 1268 [38,39], and thus toxicological investigations have been limited for this highly chlorinated mixture. While the concentrations measured in the dolphins were dominated by highly chlorinated octa- through decachlorobiphenyl congeners, mono-ortho congeners such as PCB 189 were also detected (see the electronic supplementary material) and strongly correlated with the summed 1268 congeners ($r = 0.92$). Both congener groups were significantly higher in the Georgia dolphins when compared with the two reference sites (see the electronic supplementary material), and it is likely that both congener groups contribute to the overall toxicity of the mixture.

Other persistent organic contaminants (POCs) have potential for endocrine and immune disruption and must be considered as possible confounding factors. However, concentrations of other POCs measured in the Georgia dolphins were similar or even lower than those measured from other areas of the US coast where such severe health impacts have not been observed. Concentrations of DDT and chlordane compounds in the Georgia dolphins were significantly lower than concentrations measured in one or both reference sites (see the electronic supplementary material). BDEs were higher for Georgia dolphins when compared with both reference sites, but were still lower than those measured in dolphins from many other US Atlantic coast sites [40]. Furthermore, total PCB concentrations were on average 43-fold higher than BDE concentrations in the Georgia dolphins, and the toxic mono-ortho PCBs alone were 1.6-fold higher than BDEs (see the electronic supplementary material). Therefore, while potential additive effects of the BDEs

cannot be ruled out, with such large differences in concentrations, it is unlikely that the BDEs significantly influenced the correlations between PCBs and health endpoints.

5. CONCLUSIONS

The impact of PCBs on the health and sustainability of cetacean populations has been of concern for decades, prompted initially by a series of die-offs of dolphin and other marine mammal populations near industrialized coastlines (see [41] for review). Concern is particularly warranted for odontecetes (toothed whales and dolphins), which generally occupy high trophic positions. While a number of studies have documented high PCB exposures in these species, studies to examine associated health impacts have been limited owing to the ethical and logistical constraints of studying such large aquatic mammals, which are protected under federal laws of the USA as well as many other nations (see [41] for discussion). The results of our research clearly demonstrate that bottlenose dolphins are vulnerable to PCB-related health effects, at least partially mediated through the endocrine system, and that while PCBs are no longer used in the USA they remain in marine food webs where they still pose a significant risk to upper trophic marine wildlife. By confirming a strong exposure–response relationship between PCB burden and endocrine and immune endpoints in a cetacean species, our results have important implications for risk assessment, and ultimately the conservation and management, of cetacean populations worldwide.

These findings also have significance beyond conservation of dolphins and whales. To date, the majority of PCB toxicity studies have focused on the dioxin-like congeners or more common Aroclor mixtures. Higher chlorinated congeners are generally assumed to be more inert, resistant to dechlorination and metabolism, and the highly chlorinated mixtures are, therefore, considered to have lower toxic potential. This study provides evidence that although a potentially lower toxicity, Aroclor 1268 may accumulate to sufficiently high concentrations to produce significant health impacts. This suggests a need for further study to elucidate mechanisms and potential impacts on other top-level predators, as well as human populations who regularly consume fish from the same marine waters.

This work was conducted under NMFS Permit no. 932-1905/MA-009526 issued to Dr Teresa Rowles and protocols were reviewed and approved by a NOAA/NMFS ad hoc Institutional Animal Care and Use Committee (IACUC).

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Technical Details for Laboratory Methods

Anemia, Hypothyroidism and Immune Suppression Associated with Polychlorinated Biphenyl Exposure in Bottlenose Dolphins (*Tursiops truncatus*)

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Chemical Analysis

Methods

PCBs as well as other persistent organic pollutants (POP) were measured in blubber using fluid extraction (PFE), size exclusion and alumina solid phase extraction cleanup followed by gas chromatography mass spectrometry (GC/MS) using two different capillary columns and two different ion sources. Method details are described elsewhere (Litz et al. 2007, Yordy et al. 2010, Kucklick et al. In press). Briefly, POPs including 55 PCB congeners, were extracted from samples using pressurized fluid extraction. Analytes of interest were isolated from the extract using size exclusion chromatography and alumina solid phase extraction and were quantified using gas chromatography mass spectrometry (GC/MS) (Agilent 6890/5973, Agilent Technologies, Santa Clara, CA) in the electron impact mode with selected ion monitoring. Cyclodiene compounds including chlordanes were determined by GC/MS with negative chemical ionization as described elsewhere (Litz et al. 2007).

A blank and 1 to 3 aliquots of National Institute of Standards and Technology Standard Reference Material (SRM) 1945 Organics in Whale Blubber were extracted and analyzed alongside samples. The POP concentrations determined on the aliquots of SRM 1945 agreed to within 7.5% ± 3.5% of the certified values.

The following summary variables for contaminant classes were computed by summing individual congeners/compounds; values below detection limit were set to zero:

- Total DDT: sum of 2,4'-DDE, 4,4'-DDE, 2,4'-DDD, 4,4'-DDD, 2'4-DDT and 4,4'-DDT
- Total Chlordane: sum of cis- and trans-chlordane and nonachlor
- Total BDE: sum of brominated diphenyl ether (BDE) congeners BDE 28, BDE 47, BDE 100, BDE 99, BDE 155, BDE 154, BDE 153

- Total PCB: sum of International Union of Pure and Applied Chemistry (IUPAC) Numbers 18, 28+31, 44, 49, 52, 56, 66, 70, 74, 87, 92, 95, 99, 101, 105, 110, 118, 119, 128, 130, 137,138, 146, 149, 153+132, 151, 154, 156, 157, 158, 163, 167, 170, 172, 174, 176, 177, 178, 180+193, 183, 185, 187, 189, 194, 195, 197, 199, 200, 201, 202, 203+196, 206, 207,208, and 209

Results

Chemical analysis results from the Brunswick and Sapelo samples, as well as results from prior remote biopsy sampling near Brunswick and Sapelo Island (N= 105) are described in detail by Balmer et al. (In press). These data, as well as the chemical analysis results for the Sarasota (N=12) and Beaufort (N=7) samples, and other biopsy sample data are also included in the broader contaminant spatial trend analysis by Kucklick et al. (submitted).

Chemical analysis results were stratified into two classes: 1) adult females, 2) males and non-adult females and concentrations measured from the latter group were used for comparisons among sites. Data were stratified by site and geometric means and 95% confidence intervals for the major contaminant classes were calculated by log-transforming the concentrations, calculating the mean and 95% confidence intervals for the log-transformed values, and then back-transforming the values (Figure 1).

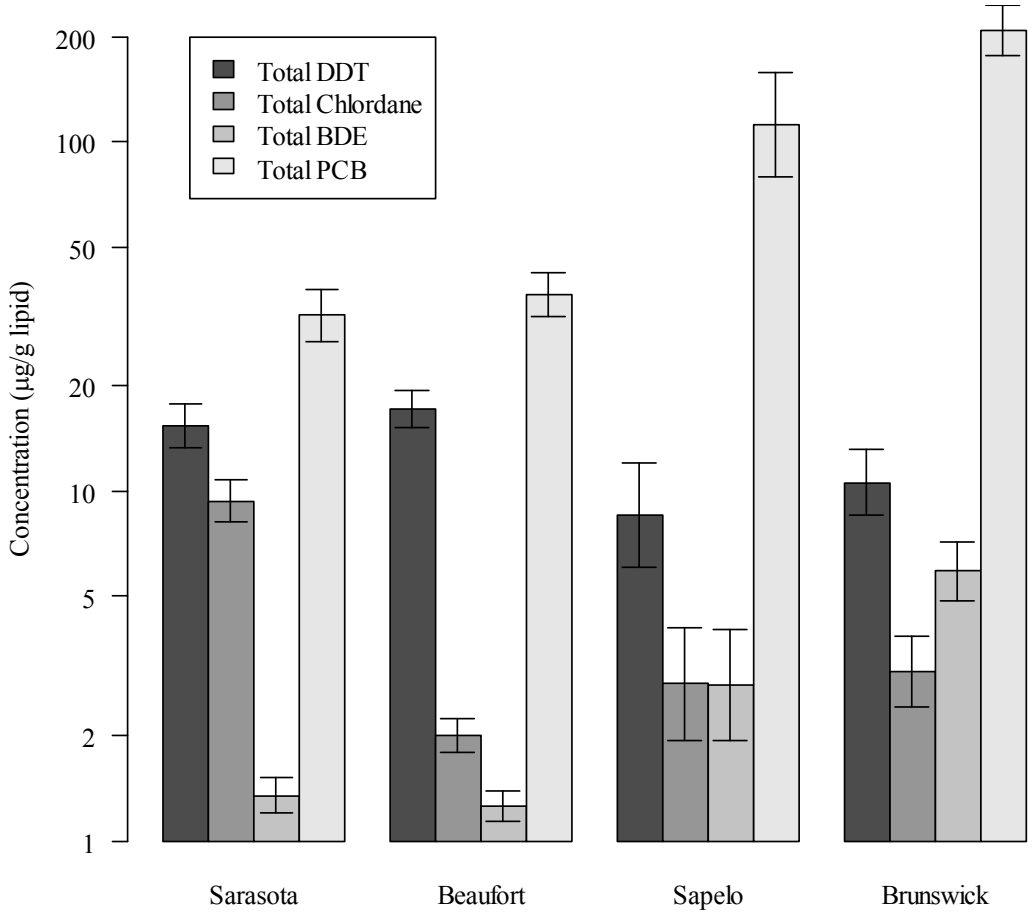

Figure 1. Concentrations of major contaminant groups measured in blubber of male and non-adult female bottlenose dolphins from 4 coastal sites: Sarasota, Florida (N=12); Beaufort, North Carolina (N=7); Sapelo Island, Georgia (N=12); and Brunswick, Georgia (N=10). Bars represent geometric mean and whiskers represent 95% confidence intervals around the mean. y-axis is a log scale. Toxaphene was not measured in Sarasota or Beaufort.

Additionally, 24 toxaphene compounds were measured in samples from Sapelo Island and Brunswick, but these compounds were not measured in previous samples from Sarasota and Beaufort. Geometric mean concentration of summed toxaphene compounds from male and non-adult female dolphins from Brunswick and Sapelo was 1.10 and 0.35 µg/g lipid, respectively.

To examine PCB congener patterns between sampling sites, 4 congener groups were considered (Figure 2):

- Aroclor 1268 congeners: PCB congeners that have been reported as primary constituents of Aroclor 1268 (Maruya and Lee 1998, Balmer et al. In press, Kucklick et al. In press) – *i.e.*, PCB 174, PCB 180+193, PCB 183, PCB 187, PCB 194, PCB 203+196, PCB 199, PCB 200, PCB 201, PCB 202, PCB 206, PCB 207, PCB 208, PCB 209, were summed.
- Mono-ortho PCB congeners: Congeners PCB 105, PCB 118, PCB 156, PCB 157, PCB 189 were summed.
- Recalcitrant congeners: PCB 153/132 and PCB 138 were summed separately for comparison. These are recalcitrant congeners that are found in many other PCB mixtures and often measured in high concentrations in environmental samples.
- Other congeners: All remaining PCB congeners were summed.

PCB concentrations measured in Sapelo and Brunswick dolphins were dominated by congeners associated with the Aroclor 1268 PCB mixture, as compared to concentrations from Sarasota and Beaufort dolphins which had relatively low concentrations of the 1268-associated congeners. Mono-ortho congeners also differed among the sites (2-factor ANOVA; site p-value<0.001; length class p-value<0.001), with highest concentrations in Brunswick and Sapelo dolphins. Mono-ortho congeners were dominated by PCB 189, a hepta-chlorobiphenyl that comprised on average 75% of the mono-ortho congeners for Brunswick and Sapelo samples.

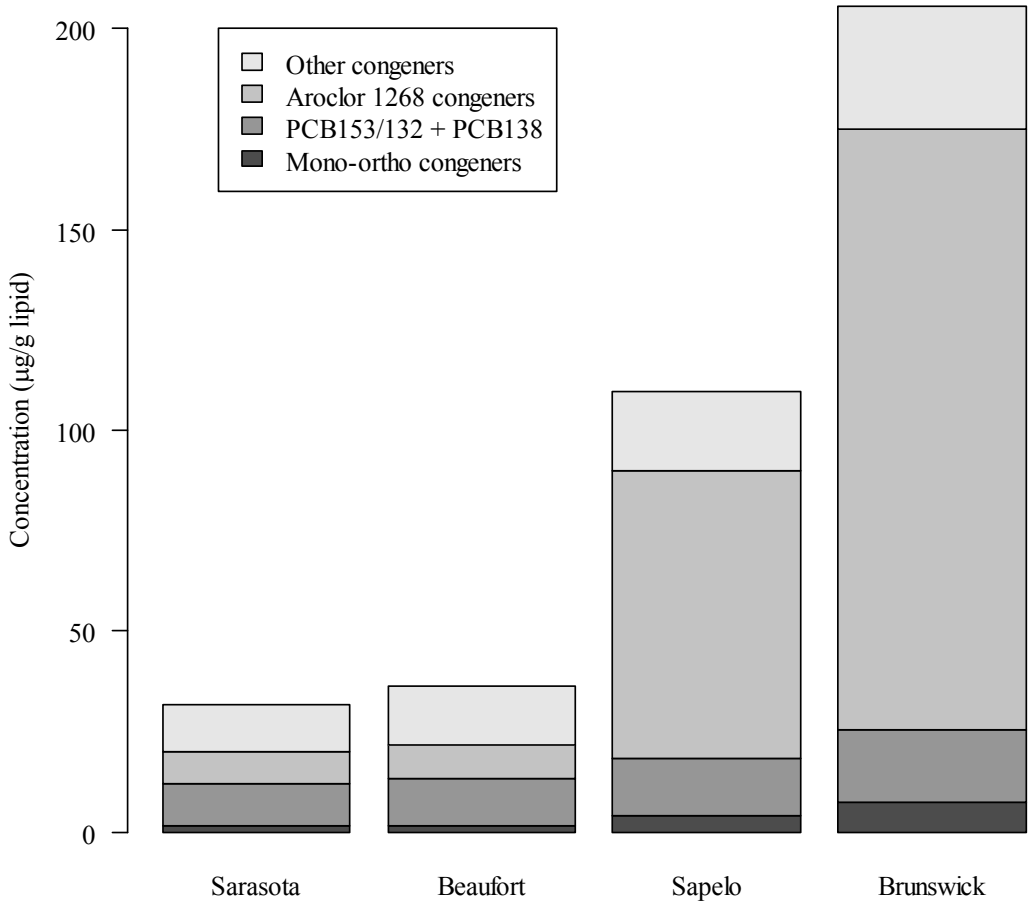

Figure 2. Composition of PCB congeners measured in blubber of male and non-adult female bottlenose dolphins from 4 coastal sites: Sarasota, Florida (N=12); Beaufort, North Carolina (N=7); Sapelo Island, Georgia (N=12); and Brunswick, Georgia (N=10).. Bars represent geometric mean concentration for each specified congener group.

Functional Immune Methods

Isolation of mouse immune cells

Adult female B6C3F1 mice (*Mus musculus*; Charles River Laboratories, MA) were maintained at 18-26°C with relative humidity between 40 and 70%, and a light/dark cycle at 12-h intervals. Animals were housed five mice per cage containing sawdust (hardwood) bedding. Blood was collected via cardiac puncture immediately after euthanasia using CO_2 inhalation, followed by cervical dislocation to ensure death, and the spleen was removed aseptically and stored in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY) until processing (below). DMEM was supplemented with (all from Gibco BRL, Grand Island, NY) 1 mM sodium pyruvate, 100 μM non-essential amino acids, 25 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, along with 10 % fetal bovine serum (Hyclone, Logan, UT), thereafter referred to as complete DMEM. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut.

From the mouse spleen, mononuclear cells were isolated by density gradient centrifugation on Ficoll-Paque plus (Amersham Biosciences, Uppsala Sweden) for 15 minutes at 720 g. The mononuclear cells were re-suspended in complete DMEM, washed twice, and enumerated with their viability assessed using the exclusion dye trypan blue.

Erythrocytes from mouse whole blood were lysed using NH_4Cl and the leukocytes were re-suspended in Hanks Balanced Salt Solution (HBSS, Gibco BRL, Grand Island, NY). Cells were washed twice with HBSS, and their viability was assessed using the exclusion dye trypan blue. Viability was typically greater than 90%.

Isolation of dolphin immune cells

Dolphin whole blood was centrifuged for 20 min at 220 g, and the buffy coat was collected and

re-suspended into complete DMEM. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Paque plus for 35 min at 990 g. The PBMCs were collected, washed once, and enumerated with their viability assessed using the exclusion dye trypan blue. Viability was typically greater than 90%.

Erythrocytes from dolphin whole blood were lysed using NH_4Cl and the leukocytes were re-suspended in HBSS. Cells were washed twice with HBSS, and their viability was assessed using the exclusion dye trypan blue. Viability was typically greater than 90%.

Neutrophil and Monocyte Phagocytosis

Briefly, the leukocyte concentration was adjusted to 2 x 10^6/mL in HBSS and plated in round bottom 96-well plates (Falcon, Becton Dickinson, Lincoln Park, New Jersey, USA), in triplicate. One μm-diameter fluorescent latex beads (Molecular Probes, Eugene, OR) were added to the cell suspension to obtain a ratio of approximately 100 beads/cell, and cells were incubated for 1 hour at 37°C, under agitation at 300 rpm using a Thermomixer R (Eppendorf, Hamburg, Germany). The cell suspension from each well was then layered on a cushion of ice cold 3% bovine serum albumin (Sigma, St. Louis, MO) and centrifuged at 150 g for 8 min at 4°C. The supernatant containing the free beads was discarded and the cells were re-suspended in 200 μl of phosphate buffered saline (PBS, Gibco, Grand Island, NY) containing 1% neutral buffered formalin (Decal Corp, Tallman, NY). Cells were stored at 4°C until analysis (within 24 hr). The fluorescence of approximately 10,000 cells was read with a FACScan (Becton Dickinson, Rutherford, New Jersey, USA) flow cytometer using the CellQuest software (Becton Dickinson Immunocytometry System, San Jose, CA). Neutrophils and monocytes were gated electronically according to their relative size (forward scatter; FSC) and complexity (side scatter; SCC). The fluorescence of the

cells was read at 530 nm (FL-1) on a logarithmic scale using the fluorescence of free beads as reference. Cells acquired a fluorescence equal to that of the number of beads they ingested.

Lymphocyte Proliferation

Briefly, dolphin peripheral blood mononuclear cells (PBMCs) or mouse splenocytes in complete DMEM were plated (2 x 10^5 cells/well) in 96 well flat bottom tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ), in triplicate. Cells were incubated at 37° C with 5% CO_2 for a total of 66 hr with a T cell mitogen (concanavalin A, ConA; Sigma, St. Louis, MO) and a B cell mitogen (lipopolysaccharide, LPS; Sigma, St. Louis, MO). ConA was used at the optimal concentration of 1.0 µg/mL. LPS was used at the optimal concentration of 5 µg/mL. Lymphocyte proliferation was evaluated as the incorporation of 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue, added for the last 18 hr of incubation, and further detected with a monoclonal antibody and a colorimetric enzymatic reaction (Cell Proliferation ELISA BrdU (colorimetric), Roche Diagnostics GmbH, Mannheim Germany) as per manufacturer's instructions using an ELISA plate reader (Multiskan EX v.1.0) at 450 nm with a reference wavelength of 690 nm.

Thyroid Hormone Analysis

Serum concentrations of total thyroxine (TT4), free thyroxine (FT4) and total triiodothyronine (TT3) were measured by solid-phase ^{125}I radioimmunoassays (RIA) using commercially available kits. The intra- and inter-assay coefficients of variation (CVs) and the analytical sensitivities according to the manufacturers are given in Table 1. Two sets of parameters are listed for TT3, because the manufacturer discontinued production of the RIA used prior to August 2006. The RIA for FT4 was preceded by equilibrium dialysis to separate bound from free T4. Every assay met all quality assurance criteria, and internal controls (canine, equine, feline, bovine) were run in each assay.

Table 1. Manufacturers and performance data for RIAs used to measure thyroid hormones.

Hormone	Manufacturer	Sensitivity	Intra-assay CV	Inter-assay CV
TT4	Siemens[1]	0.25 μg/dL	3.2%	6.1%
FT4	Antech[2]	0.15 ng/dL	11.5%	15.2%
TT3	DPC[3]	0.08 ng/mL	5.3%	7.4%
	Siemens[4]	0.07 ng/mL	5.5%	7.6%

[1]Siemens Healthcare Diagnostic Inc, (Los Angeles, CA), Coat-A-Coat® Total T4 (TKT41)
[2]Antech Diagnostics, (Irvine, CA), Free T4 by Equilibrium Dialysis (#30-0640V)
[3]Diagnostic Product Corp, (Los Angeles, CA). Coat-A-Coat Total® Canine T3 (TKC31)
[4]Siemens Healthcare Diagnostic Inc, (Los Angeles, CA), Coat-A-Coat® Total T3 (TKT31)

TT3 assay comparison

Internal controls (canine, equine, bovine) routinely analyzed in all TT3 assays performed at the Diagnostic Endocrinology Laboratory, Animal Health Diagnostic Center, Cornell University were used to compare TT3 values from the Canine T3 and Total T3 assays. Duplicate samples were run using both assays and regression analysis of values from the Canine T3 assay versus the Total T3 assay was conducted using (Statistica© 9.0, StatSoft Inc.). Data were log-transformed prior to analysis to meet model assumptions. The TT3 measures between the two assays were highly correlated (Figure 3, $r^2=0.9971$, $p<0.001$), although the Canine T3 measures were

consistently higher. Therefore, the estimated regression equation (Figure 1) was used to adjust TT3 values that had previously been determined using the Canine T3 assay.

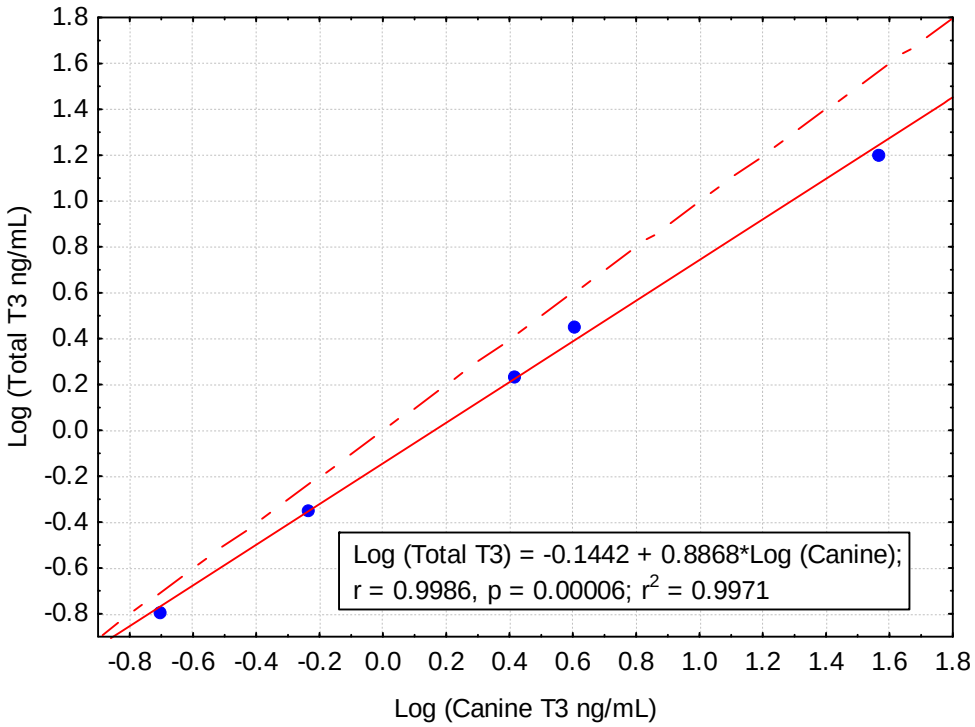

Figure 3. Regression of Canine T3 Assay and Total T3 Assay measures. Each of the data points represents the mean of 21 replicate measurements on 5 samples (2 canine, 2 equine, and 1 bovine). The dashed line (45°) represents perfect concordance; the solid line is the fitted regression equation.

Data Analysis

Dolphins were categorized into age classes by applying the following criteria (Schwacke et al. 2010):

Adult ≥ 10 years, or if age not determined then ≥ 240 cm

Subadult ≥ 2 and < 10 years, or if age not determined then ≥ 200 and < 240 cm

Calf < 2 years, or if age not determined then < 200 cm

The number of dolphins captured and examined, stratified by site and age class, are shown below in Table 2.

Table 2. Number of dolphins captured and examined, stratified by site and age class. ND=not determined.

Age Class	Sampling Area	No. Examined	Age Range
Adult Female	Sapelo Island, GA	2	10-36
	Brunswick, GA	3	22-29
Adult Male	Sapelo Island, GA	6	16-27
	Brunswick, GA	7	11-32
Subadult	Sapelo Island, GA	5	ND
	Brunswick, GA	4	ND
Calf	Sapelo Island, GA	1	ND
	Brunswick, GA	1	ND

Statistical method for removal of outliers

Data were examined for outlying values and extreme outliers were removed prior to analysis. Extreme outliers were assessed as values that were greater than the upper quartile plus 1.5 times the quartile range or less than the lower quartile minus 1.5 times the quartile range. There were 1, 2, and 3 data points omitted from the analysis as outliers for TT3, FT4 and neutrophil phagocytosis index, respectively.

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