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The effects of the organopollutant PCB 126 on bone density in juvenile diamondback terrapins (*Malaclemys terrapin*)

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ABSTRACT

Bone is a dynamic tissue with diverse functions including growth, structural support, pH balance and reproduction. These functions may be compromised in the presence of organopollutants that can alter bone properties. We exposed juvenile diamondback terrapins (*Malaclemys terrapin*) to 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), a ubiquitous anthropogenic organochlorine, and measured organic content, apparent bone mineral density (aBMD) using radiography and computed tomography, and quantified bone microstructure using histological preparations of femora. PCB-exposed terrapins were smaller in total size. Skulls of exposed animals had a higher organic content and a skeletal phenotype more typical of younger animals. The femora of exposed individuals had significantly reduced aBMD and significantly more cortical area occupied by non-bone. Because bone is an integral component of physiology, the observed skeletal changes can have far-reaching impacts on feeding and locomotor performance, calcium reserves and ultimately life history traits and reproductive success. Additionally, we caution that measurements of bone morphology, density, and composition from field-collected animals need to account not only for relatedness and age, but also environmental pollutants.

1. Introduction

Bone is a metabolically active tissue which is deposited and resorbed throughout an organism's life. This tissue provides strength for the skeleton to support the body during locomotion and mechanical strength during activities such as chewing. Bone serves as a physiological reservoir of important biomolecules including carbonate mobilized from bones to buffer against acidosis during prolonged submersion in water (Jackson, 2000) and calcium to aid in ovogenesis (De Buffrenil and Francillon-Viellot, 2001). For these reasons, any alteration in bone density, compactness, or composition could have significant consequences on the animal's behavior, physiology, and fitness.

Many environmental pollutants are known to disrupt the endocrine system and thus an emerging consequence of pollutant exposure is its effect on bone. Early studies suggested water pollution was the leading cause of fish skeletal deformities and Mehrle et al. (1982) provided some of the first evidence correlating pollutant exposure (polychlorinated biphenyls [PCBs] and metals) with weakened bones. Bergman et al. (1992) and Mortensen et al. (1992) correlated lesions and bone loss in seal skulls with a higher incidence of organochlorine contaminant exposure. However, in the only published study of organochlorine effects on reptilian bone, alligators from the contaminated Lake Apopka, Florida site had greater trabecular and total bone mineral density than individuals from a nearby reference site (Lind et al., 2004). Thus, environmental contaminants may exhibit species-specific effects.

Fewer studies have used laboratory exposures of PCBs or related dioxins to experimentally test the effects of organopollutants on bone biology. Laboratory studies exposing fish to dioxin (2,3,7,8-TCDD) have documented shortened or deformed jaws (e.g. Elonen et al., 1998; Heiden et al., 2009; Teraoka et al., 2002) and Xiong et al. (2008) explored the morphological and molecular mechanisms underlying similar changes in zebrafish. Experimental exposure to 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) caused osteolysis in mink (Mustelidae) mandibles (Render et al., 2000), but similar effects were not seen in rats (Aulerich et al., 2001). However, female rats exposed to PCB 126 or TCDD experienced changes in long bone (humerus and femur) torsional strength, geometry and composition (Lind et al., 2000; Miettinen et al., 2005).

We investigated if Diamondback terrapins (*Malaclemys terrapin* Schoepff [1793]) experimentally exposed to PCB 126 exhibit reduced apparent bone density and altered bone microstructure in skeletal elements. Diamondback terrapins are a brackish water turtle found in coastal salt marshes, estuaries and tidal creeks along

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the Eastern and Gulf Coasts of North America. Many of these habitats are susceptible to contamination by anthropogenic toxicants such as PCBs (Ko and Baker, 2004) and petroleum hydrocarbons from onshore and offshore drilling and transportation accidents (Collins et al., 2003; USFWS, 2011). Thus, insights into bone physiology in response to organochlorines of this sentinel species will shed light on reptilian as well as vertebrate ecophysiology and ecotoxicology.

2. Methods and materials

In June 2002, eleven clutches of recently laid (less than 24 h) diamondback terrapin eggs were collected from the western shores of the Patuxent River, Chesapeake Bay (MD, USA). Eggs were transported to Ohio University and incubated at male producing temperatures (sex confirmed at sacrifice by dissection). Only those individuals exhibiting normal growth were included in the present study. All procedures outlined were conducted in accordance with the guidelines of the Ohio University Institutional Animal Care and Use Committee (#L02-06).

The experimental methods followed those of Holliday et al. (2009). In brief, forty-four juvenile turtles were randomized and either received an intraperitoneal injection of 20 μg/g PCB 126 (Ultra Scientific, Kingstown, RI, USA) dissolved in dimethylsulfoxide (DMSO; Fisher Scientific, Pittsburgh, PA, USA) and corn oil (7% final DMSO concentration) or a sham injection. The PCB dose was based on previous experiments using male terrapins of similar age and held under nearly equivalent laboratory conditions (Ford, 2005) and the intraperitoneal dosages chosen by Yawetz et al. (1998). Although the PCB concentration administered in the present study was higher than tissue concentrations reported from wild terrapins (Kannan et al., 1998), Stone et al. (1980) reported far higher concentrations of total PCBs in the livers of common snapping turtles (*Chelydra serpentina*). Furthermore, similar congeners have been administered via intraperitoneal injections to examine biochemical processes in turtles (Yawetz et al., 1998) and growth and metabolic rates in terrapins (Holliday et al., 2009) producing results still relevant to wild populations. Because our previous studies did not show a significant effect of the DMSO/corn oil vehicle on hatchling growth or survival (Ford, 2005; Holliday et al., 2009) and studies with other turtles similarly showed no effect of corn oil on biochemical reactions (Brown et al., 2002), terrapins in the uncontaminated (0 μg/g) treatment received a sham (empty syringe) injection. Turtles were housed individually at 28 °C (±2 °C) in 12.5 cm × 17.5 cm × 6 cm plastic containers in approximately 300 ml of water under a full-spectrum Reptisun® (Zoo Med, San Luis Obispo, CA, USA) 12:12 light cycle in a walk-in environmental chamber. Twice a week turtles were fed two tablespoons of Kordon® frozen brine shrimp (Novalek, Hayward, CA, USA) and 24 h later their water was completely changed. All turtles ate throughout the study and no mortality was observed. Six months after PCB exposure (turtle age = 14 months) the animals were euthanized. Each turtle was dissected and the organs removed. Specimens were pinned in anatomical position and dried to constant mass in a Fisher Scientific Isotemp® 500 Series drying oven to remove excess fluids that might interfere with imaging.

To determine bone mineral content, eight terrapin skulls ($n = 4$ PCB-exposed and $n = 4$ unexposed) were dried to constant mass at 75 °C, and then ashed at 600 °C for 12 h. Organic content was calculated as (dry weight − ash weight)/wet weight. Because initial skull lengths were significantly different ($p < 0.001$), ash weight and organic content were corrected for size by dividing by average skull length. These data were then compared between PCB and unexposed terrapins using a *t*-test in NCSS (Kaysville, UT, USA).

All forty-four terrapins, including the 8 destructively sampled above, were radiographed ventrodorsally using a Hewlett-Packard Faxitron soft X-ray machine (30 kvp, 2.75 mA, duration = 180 s, film-to-source distance = 122 cm) and Kodak Industrex M film contained in lead-backed cardboard cassettes which was then manually developed in Hale Medical Systems Developer 114B. Films were scanned as 1000 dpi grayscale (256) with a reference calibration phantom on a Microtek color scanner with transparency adapter. Individual images were calibrated in ImageJ (NIH) so that air was given a value of 0 and the densest bone a value of 100. Mean pixel intensity was used as a proxy for apparent bone mineral density (aBMD) and was measured at (1) 10 × 10 pixel regions of interest in the right hyoplastron anterior to, at, and posterior to the first ossification center and (2) the pixels along the length of the midshaft of the 4th right and left ribs distal to the rib's articulation with the vertebrae to midway along the length of the shaft. Values for aBMD were compared between PCB-exposed and unexposed turtles using a *t*-test. Pixel intensity of the carapace could not be measured because the lack of significant ossification made the costal elements largely indistinguishable from air.

Fifteen of the radiographed terrapins were subsequently scanned using a GE eXplore Locus Small Animal MicroCT scanner at Ohio University, Athens, OH. The skulls and caudal halves of specimens were scanned using a short scan (180°) at 80 kVp and 450 mA at slices of 0.45 μm. All scans were conducted using a phantom to calibrate Hounsfield units (HU) for bone, air and water. Image data were exported in DICOM format and reconstructed in Amira 4.0 (Visage Imaging) for additional study. Datasets were manually cropped in Amira to contain only the region of interest (i.e. whole skull, mandible, and femur). Hounsfield units, standard units of radioopacity (Brooks, 1977), were used as a proxy for apparent bone mineral density (aBMD). Voxel values were divided into three bins: 850–1733 HU low density bone, 1734–2617 HU medium density bone, and 2618–3500 HU high density bone. Because the regions of interest (skull and femur) also contained unwanted elements, the voxels of the entire datasets were first thresholded and then unwanted elements in the ROI were manually deselected. Percent bone volume was calculated for each bin (bin voxel volume/total volume) because this measurement diminishes the effect of body size in the analysis. Mean aBMDs were compared between PCB-exposed and control animals using a *t*-test.

Ten of the radiographed turtles ($n = 5$ PCB-exposed; $n = 5$ unexposed) were examined for histological differences in bone microstructure. Diaphyses of right femora were removed, fixed in neutral-buffered formalin, decalcified in Cal-Ex (Fisher Scientific) and embedded in paraffin. Five μm-thick sections were stained with Mallory's trichrome, viewed and captured at 40× using an Aperio ScanScope CS slide scanner, and imported into ImageJ for analysis. In ImageJ, all images were calibrated, converted to 8-bit grayscale and a black-white threshold applied to distinguish bone from void area (*sensu* Parfitt et al., 1987) which includes all non-bone areas in the medullary space and cortical bone. We measured total cortical area, medullary area and the total area of the cortical void space using the analyze particle function. Porosity or percent area of bone that was occupied by cortical void was calculated as (cortical void area/cortical area) × 100. Unexposed and PCB-exposed groups were compared using a *t*-test.

3. Results

Four uncontaminated individuals and three that received the 20 μg/g PCB 126 exposure were randomly selected and sent to S.D. Holladay (Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA, USA). Hepatic concentrations as determined by gas chromatography with electron capture detection

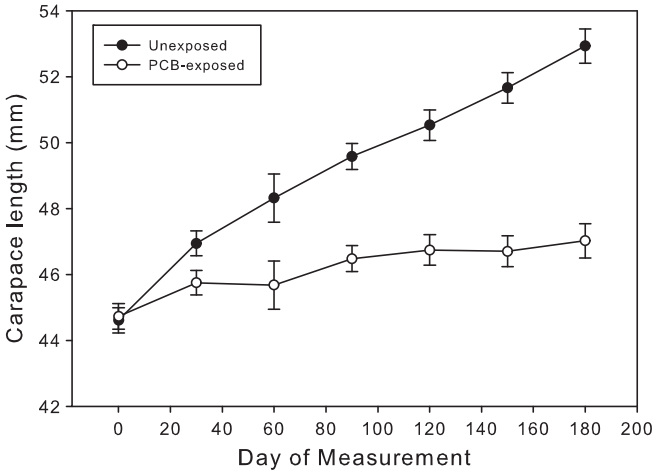

Fig. 1. Diamondback terrapins exposed to PCB 126 were smaller in carapace length than same-aged individuals that were not exposed (modified from Holliday et al., 2009).

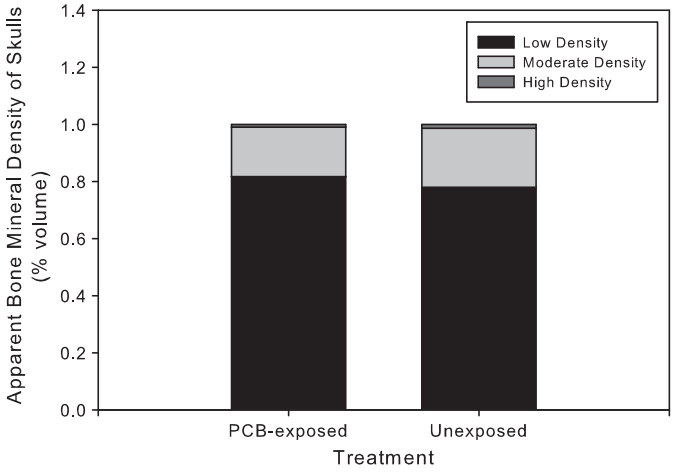

Fig. 2. Diamondback terrapins in both unexposed and PCB 126 exposed treatment groups did not differ in the apparent bone mineral density of skulls.

Table 1
Apparent bone mineral density (aBMD) from radiographs of plastrons and ribs comparing PCB-126 exposed and unexposed diamondback terrapins. Values are mean pixel intensity and standard deviations.

	PCB-126 exposed	Unexposed	*p*-Value
Plastron aBMD	40.73 (7.64)	39.48 (8.00)	0.61
Rib aBMD	13.59 (4.27)	13.13 (5.02)	0.75

were less than 0.03 μg/g and averaged 13.2 μg/g (wet wt; detection limit = 0.02 μg/g) in uncontaminated and PCB-exposed terrapins respectively.

Turtles exposed to PCB 126 were significantly smaller in length (Fig. 1) and mass (see Holliday et al., 2009) than unexposed animals. There was a significant difference in skull ash mass between turtles exposed to PCB 126 and unexposed individuals (PCB $\bar{x} = 0.659 \pm 0.01$ g, unexposed $\bar{x} = 0.984 \pm 0.02$ g, $p = 0.048$); however, this difference ceased to exist when skull ash mass was corrected for size ($p = 0.11$). The size-corrected organic content of PCB 126 exposed turtles had a significantly higher organic content than unexposed turtles (PCB $\bar{x} = 0.186 \pm 0.01$ g/cm; unexposed $\bar{x} = 0.156 \pm 0.005$ g/cm, $p = 0.002$). Apparent X-ray BMD of the hypoplastron ($p = 0.61$) and 4th rib ($p = 0.75$) were not significantly different between PCB-exposed and unexposed terrapins (Table 1). Although total skull and mandibular μCT aBMDs were not significantly different (Table 2 and Fig. 2) some patterns did exist.

Qualitatively, the terrapin skulls shared two common patterns of mineralization. First, the facial skeleton, palate, and dentary were denser than the neurocranium in all specimens. Second, the skull areas associated with the ear, including the paired otoliths and the incisure columella auris, the portion of the quadrate forming which transmits the columella (Gaffney, 1979), were among the densest cranial structures among all individuals (Fig. 3). The femoral epiphyses within the control and treatment groups showed the greatest difference in distribution of dense bone where the exposed turtles tended to have less dense epiphyses.

Table 2
p-Values for apparent bone mineral density (aBMD) from μCT data comparing high density, moderate density and low density bone in complete skulls, mandibles and femora of PCB-exposed and unexposed diamondback terrapins.

	High density	Moderate density	Low density
Total skull	0.1917	0.1955	0.2973
Mandible	0.1681	0.1503	0.4648
Femur	0.001*	0.003*	0.011*

* Only femoral aBMDs were significantly different.

The aBMD of femora, as determined by μCT, differed significantly between treatments. PCB-exposed individuals had significantly more low density bone than unexposed animals ($p = 0.001$; Table 2 and Fig. 4) which had significantly more moderate density ($p = 0.003$) and high density ($p = 0.011$) bone. In the femora, the densest bone was found in the diaphysis (Fig. 3). PCB turtles had significantly smaller femoral length (PCB $\bar{x} = 0.096 \pm 0.06$ mm, unexposed $\bar{x} = 1.12 \pm 0.04$ mm), $p < 0.0001$) as expected due to their overall small body size (Fig. 1).

Histological analyses revealed no significant differences in femoral cortical area ($p = 0.12$) or marrow area ($p = 0.77$). However, much of the cortical area in PCB-exposed turtles was not occupied by bone, but instead had a significantly larger void area (PCB $\bar{x} = 24.3 \pm 6.04\%$, unexposed $\bar{x} = 13.0 \pm 5.04\%$, $p = 0.018$; Fig. 5).

4. Discussion

Polychlorinated biphenyls are physiologically active chemicals (Safe, 1994) with known endocrine disrupting effects (e.g. Gregoraszczuk et al., 2003). As such, exposure to PCB 126 and similar organochlorine contaminants has been shown to affect bone formation via the aryl hydrocarbon receptor (Singh et al., 2000; Xiong et al., 2008) and osteoblast estrogen receptors (Eriksen et al., 1988; Onoe et al., 1997). On the other hand, PCB exposure can induce a stress response which, through the production of glucocorticoids, can lead to bone loss through osteoclastic activity or osteocyte apoptosis (Van Staa, 2006) and has been shown to directly affect bone resorption markers in rats (Ramajayam et al., 2007).

Interestingly, changes in bone microstructure following organochlorine exposure manifest themselves differently among vertebrate species. Field studies have correlated exposure to organochlorines with loss of alveolar bone in grey seals (*Halichoerus grypus*; Bergman et al., 1992), reduced bone density in deer mice (*Peromyscus maniculatus*; Johnson et al., 2009), and altered mineralization in clapper rails (Aves: *Rallus longirontris*; Rodriguez-Navarro et al., 2006). Laboratory studies have begun to provide insight on the mechanisms underlying these changes. Rats experimentally exposed to PCB 126 tended to have significantly higher organic content in their tibiae (Lind et al., 1999) and humeri (Lind et al., 2000), results similar to those found in the skulls of terrapins in the present study. Lind et al. (2000) showed the increased organic content in rat long bones was the result of the disruption

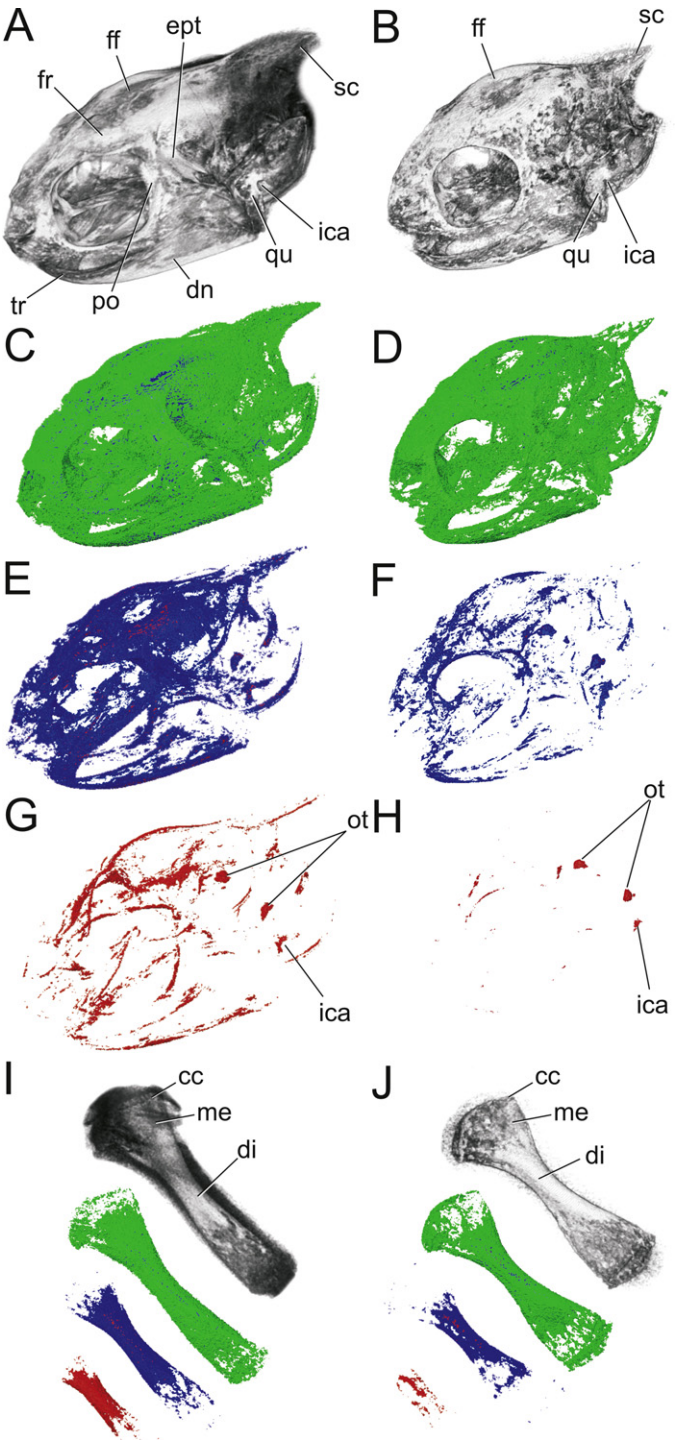

Fig. 3. Patterns of bone mineral density in the skulls and femora of two representative specimens of diamondback terrapins scaled to the same size. (A, left) Rendered visualization of the skull of specimen G6 (control group) in left, oblique view; (B, right) specimen U6 (treatment group); (C and D, green) segmented volume of low-density bin; (E and F, blue) segmented volume of mid-density bin; (G and H, red) segmented volume of high-density bin; (I) rendering and segmented bone density bins of the left femur of specimen G6, colors as in (A–H); (J) left femur of specimen U6. *Abbreviations*: cc, calcified cartilage; di, diaphysis; dn, dentary; ept, epipterygoid; ff, frontal fontanelle; fr, frontal; ica, incisura columnellae auris; me, metaphysis; ot, otolith; po, postorbital; qu, quadrate; sc, sagittal crest; tr, tomial ridge.

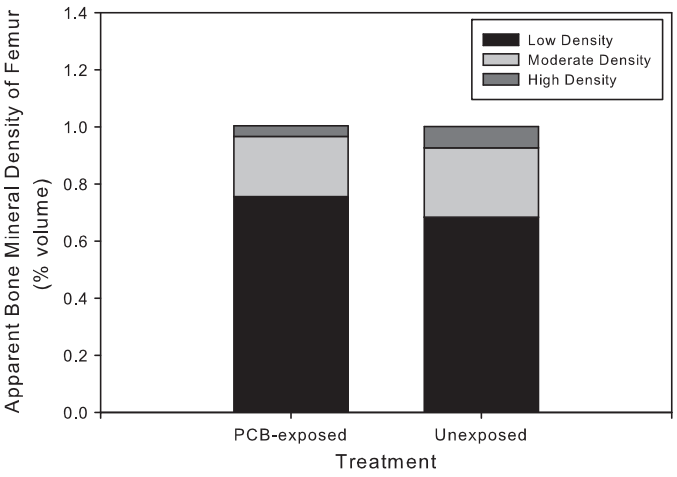

Fig. 4. Femora of diamondback terrapins exposed to PCB 126 had a larger proportion of low density bone and a smaller proportion of high density bone as compared with unexposed same aged individuals.

of mineralization via decreased amounts of hydroxyproline and collagen, but increased pyridinoline collagen cross links in rat femora. In zebrafish, TCDD blocked a chondrogenic transcription factor (Xiong et al., 2008) and interfered with cartilage formation (Teraoka et al., 2002). Decreased chondrogenesis and subsequent ossification may explain the decreased overall size of terrapins in the present study. Additionally, alterations in bone manifest differently within a bony element. For example, Gutleb et al. (2009) found PCBs affected cortical bone more than trabecular bone in the long bones of sheep. Similarly, alligators exposed to pesticides had increased density at the femoral metaphysis, but not the diaphysis suggesting within-element differences in density effects (Lind et al., 2004).

The present study, however, did not find significant differences in aBMD of the terrapin skull or mandible, although PCB 126 exposure resulted in significantly reduced femoral aBMD. PCB-exposed turtles had shorter femora as expected due to their smaller overall body size. Total cortical area and the size of the marrow cavity at the diaphysis were not affected by PCB exposure. However, the femora of turtles exposed to PCB 126, had significantly more cortical area occupied by voids. This suggests that PCB exposure may increase osteoclastic activity which agrees with recent data showing an increase in tartrate resistant acid phosphatase (TRAP) activity in Wistar rats exposed to a commercial mixture of PCBs (Ramajayam et al., 2007). However, a larger cortical area occupied by voids may also suggest a failure of osteogenesis. This hypothesis is supported

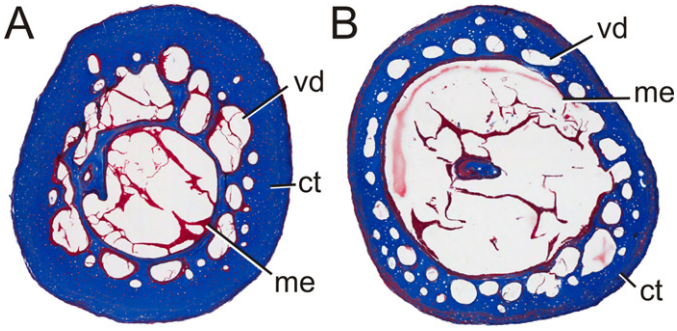

Fig. 5. Midshaft cross-sections of *Malaclemys terrapin* femora, stained with Mallory's trichrome, scaled to similar sizes. (A) Control group, specimen P2; (B) treatment group, specimen M8. ct, cortical bone; me, medullary cavity; vd, void space. Femora of terrapins exposed to PCB 126 had significantly more area occupied by void than similarly aged unexposed individuals.

by *in vitro* data from rats showing 2,3,7,8-TCDD inhibits osteoblast function (Geirthy et al., 1994). Additional mechanistic data are needed from across species to help elucidate these results.

Although all animals tended to have similar distributions of denser bone (e.g. the facial skeleton was denser than the braincase) overall, the animals exposed to PCB 126 possessed more juvenile cranial characteristics than the unexposed group. The PCB-exposed animals tended to have larger frontal-parietal fontanelles, weakly developed nuchal crests, less-pronounced tomial ridges of the maxillae and dentaries, smaller jaw muscle attachments, and generally less-dense elements of the masticatory apparatus. These features all corroborate the overall decreased growth the group experienced, mimicking features expected to be seen in less-developed or younger animals. From a biomechanical perspective less dense bone in the masticatory apparatus, as well as in the hindlimbs, can have significant impact on the individual's feeding and locomotor performance, potentially decreasing its ability to forage on harder food items, defend itself, carry itself onto basking substrates, flee predators, or maintain buoyancy.

The smaller body size, differences in cranial form, lower aBMD and increased cortical void area in femora of PCB-exposed individuals can all have far-reaching physiological and behavioral effects. Turtles mobilize calcium from bones to aid in the shelling of eggs (De Buffrenil and Francillon-Viellot, 2001). A disruption in bone modeling and remodeling caused by PCBs or other organochlorines could result in less calcium available for egg shelling thus affecting water uptake, size at hatching and residual yolk quantities (Booth, 2002). Additionally, alterations in bone mineralization processes can affect carbonate. Birds collected from a site contaminated by a PCB 126 mixture (Aroclor 1268) exhibited higher Ca/P ratios and lower carbonate and acid phosphate content (Rodriguez-Navarro et al., 2006). A reduction in available carbonate can decrease diving ability (Jackson, 2000) and thereby alter many aspects of behavior and ecology of all age classes. Finally, bone microstructure and porosity (i.e. compactness) have been shown to vary among aquatic vertebrates with different habitats where shallow-water turtles, such as terrapins, tend to posses intermediately porous diaphyses compared to the dense bones of terrestrial turtles and the highly porous bones of deep diving species (Kriloff et al., 2008). Therefore, any pathological change in the porosity of limb elements may have detrimental effects on the animal's ability to maintain proper buoyancy.

Polychlorinated biphenyls and other organopollutants have been shown to have detrimental effects on bone biology. Because of the myriad of effects, researchers need to be wary of sampling populations without prior investigation into pollutants. Regardless of contamination, we also observed a diversity of bone densities within our control group, warranting further investigation into osteogenic variation, as well as the impacts of anthropogenic pollutants on the dynamics of skeletal development, plasticity, and maintenance in turtles and other reptiles.

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