

January 2006

Effect of In Ovo Exposure to PCBs and Hg on Clapper Rail Bone Mineral Chemistry from a Contaminated Salt Marsh in Coastal Georgia

Alejandro Rodriguez-Navarro
Universidad de Granada

Christopher S. Romanek
University of Georgia

Pedro Alvarez-Lloret
University of Georgia

Karen F. Gaines
Eastern Illinois University, kfgaines@eiu.edu

Follow this and additional works at: http://thekeep.eiu.edu/bio_fac

 Part of the [Biology Commons](#)

Recommended Citation

Rodriquez-Navarro, Alejandro; Romanek, Christopher S.; Alvarez-Lloret, Pedro; and Gaines, Karen F., "Effect of In Ovo Exposure to PCBs and Hg on Clapper Rail Bone Mineral Chemistry from a Contaminated Salt Marsh in Coastal Georgia" (2006). *Faculty Research & Creative Activity*. 40.

http://thekeep.eiu.edu/bio_fac/40

This Article is brought to you for free and open access by the Biological Sciences at The Keep. It has been accepted for inclusion in Faculty Research & Creative Activity by an authorized administrator of The Keep. For more information, please contact tabruns@eiu.edu.

Effect of In Ovo Exposure to PCBs and Hg on Clapper Rail Bone Mineral Chemistry from a Contaminated Salt Marsh in Coastal Georgia

ALEJANDRO B. RODRIGUEZ-NAVARRO,^{*,†}
CHRISTOPHER S. ROMANEK,^{‡,§}
PEDRO ALVAREZ-LLORET,[†] AND
KAREN F. GAINES[‡]

Departamento de Mineralogía y Petrología, Universidad de Granada, 18002 Granada, Spain, Savannah River Ecology Laboratory, University of Georgia, Drawer E, Aiken, South Carolina 29802, Department of Geology, University of Georgia, Athens, Georgia 30602, and Department of Biological Sciences, Eastern Illinois University, Charleston, Illinois 61920-3099

The effect of Hg and PCBs (Aroclor 1268) on bone characteristics was investigated in a population of Clapper Rails (*Rallus longirostris*) inhabiting contaminated and unimpacted estuarine marsh systems in coastal Georgia. Exposure to contaminants did not affect the length or weight of leg bones, but it significantly altered the chemical composition of the bone. Specifically, bone in the contaminated site had a higher Ca to P, and lower carbonate and acid phosphate content. These characteristics are typical of more mature bone mineral and indicate that toxicants have accelerated bone maturation. FTIR spectroscopy data revealed a dose dependent change in the crystallinity of bone mineral, and the relative proportion of specific PO₄ groups in different molecular environments in the bone, with toxicant loads. These changes are most probably related to a hormonal alteration of the rate of bone remodeling induced by exposure to toxicant loads.

Introduction

Bone is a metabolically active calcified tissue in constant remodeling (1). It is composed of an organic matrix (mainly collagen type I (>90%)) and a mineral component (nanocrystalline carbonate hydroxylapatite) in proportions varying with age and location within the skeleton. Stoichiometric apatite has a Ca to P molar ratio of 1.67, while bone mineral is slightly lower mainly due to ionic substitution (Na, Mg, HPO₄ (acid phosphate), and CO₃ ions). As bone matures, bone mineral becomes more crystalline, its Ca to P ratio increases and its content of carbonate and acid phosphate decreases. Also, there is a progressive decrease in the rate of bone turnover with age. Consequently, the proportion of mature and well-crystallized mineral to newer, less crystalline, mineral varies with time and also with health condition (1, 2).

Bone mineralization is regulated by a complicated array of feedback processes which can be altered by different

genetic, endocrine, and environmental factors, resulting in abnormal or pathological composition of bone (1, 2). There is an increasing interest in medical (1, 2) and toxicological research (3–10) related to the factors that alter bone mineralization. From toxicology studies, it is well-known that environmental pollutants (e.g., heavy metals, organochlorines) cause skeletal defects and malformations in laboratory models and wild animals (3–10). Recent studies indicate that heavy metals may exert both direct and indirect controls on bone turnover. Hg, Pb, and Cd are nephrotoxic, and are known to affect Ca metabolism; for instance, kidneys produce the active form of vitamin D, which is involved in Ca homeostasis (4). They can also directly affect bone turnover by altering osteoblast and osteoclast function (5, 11). On the other hand, bone metabolism is both estrogen- and androgen-dependent (i.e., after menopause or ovariectomy there is accelerated bone loss caused mainly by estrogen deficiency) (7, 9). Exposure to organochlorines (e.g., DDT, PCB, dioxins), which are endocrine disruptors can negatively affect bone formation and composition (6–7, 9–10, 12–14). In laboratory experiments, ovariectomized rats exposed to PCBs (PCB 126) resulted in a decrease in bone length and an increase in bone mineral density (7). In this case, PCB exposure reversed the effects expressed by estrogen deficiency after ovariectomy (i.e., decreased bone density). In other studies, exposure to PCBs showed the opposite effects and produced a decreased bone mineral density and other bone pathologies (e.g., tooth loss) (6, 9, 13, 15).

Both Hg and the PCB Aroclor mixture 1268 exist in relatively high concentration at a contaminated salt marsh in coastal Georgia in the Brunswick area contaminated from decades of chemical perturbations (16–19). Specifically, this contamination has originated from waste disposal from a mercury-cell-type chlor-alkali plant and other industries that were operating at this site in the past (16). Due to their elevated levels of contamination, the site known as Linden Chemicals and Plastics (LCP) has been classified as a Superfund site. To investigate the proximate and ultimate effects of these co-contaminants on resident organisms in this site ecosystem, Clapper Rails (*Rallus longirostris*) were chosen for study as this species has well-known foraging habits, strong site fidelity, and relatively small home ranges (typically <5 ha.) (20). Clapper Rails feed exclusively on benthic organisms in salt marshes, primarily crabs, crayfish, mollusks, worms, and other marine organisms (21, 22). Through these feeding habits, individuals within the LCP marsh accumulate significant amounts of Hg and Aroclor 1268 associated with sediments (23).

A previous study of Clapper Rails inhabiting this marsh system showed that females produced eggs having shells that were abnormally thin and brittle, with altered microstructure (24). These findings suggest that Hg and/or Aroclor 1268 may have affected Ca metabolism. If this was the case, it is highly likely that bone metabolism, which is dependent on Ca-metabolism, was altered as well. The effects of contaminants potentially affecting bone should be more pronounced in newborn hatchlings. Developing and young organisms are typically more sensitive to the toxic effects of pollutants, as well as altricial species, which are often more sensitive than precocial species (4). To test this hypothesis, Clapper Rail eggs from the LCP contaminated and unimpacted Brunswick sites were collected, incubated, and hatched. Leg bones characteristics (bone dimensions and chemical composition) from hatchlings were compared to evaluate the effects of prenatal exposure to contaminants on bone formation.

* Corresponding author phone: +34 958 240059; fax: +34 958 243368; e-mail: anava@ugr.es.

[†] Dpto. Mineralogía y Petrología, Universidad de Granada.

[‡] Savannah River Ecology Laboratory, University of Georgia.

[§] Department of Geology, University of Georgia.

[‡] Eastern Illinois University.

Materials and Methods

Two adjacent study areas were chosen for this study:

Contaminated Marsh—LCP. This site is a salt marsh contaminated primarily with Hg and the PCB Aroclor 1268 at a high priority Superfund site (Linden Chemicals and Plastics; LCP) in Brunswick, Georgia. At this site, Hg and Aroclor 1268 have been found in elevated levels in the sediments and resident fauna (crabs, fish, birds) (16–19, 23).

Reference Marsh—Blythe Island. A reference marsh “Blythe Island”, located approximately 5 km from the LCP site that was not directly contaminated was chosen as a close analogue to the LCP site based on similar vegetation, tidal influence, tidal creek diversity, and water chemistry (20, 23). Similarity between sites other than contaminant levels should allow the identification of effects that those contaminants from LCP had on birds.

Clapper Rail eggs were collected from nests from the LCP and Blythe Island locations during the nesting season in March to June 2000. In total 161 eggs (from 21 nests) were collected at LCP site and 90 (from 14 nests) at Blythe Island. They were incubated at 37 °C and 95% relative humidity in an incubator (Humidaire incubator separate hatcher, Humidaire incubator Co.) and rotated automatically every 12 h for 21–23 days until hatching. For eggs that did hatch, within 48 h, chicks were observed for behavior abnormalities and then sacrificed and stored in a freezer for later analysis.

Twenty hatchlings each from the LCP and Blythe Island sites were selected for the study. Bones (femur, tibia, and fibula) were removed from one leg of each animal using a scalpel. They were rinsed with MilliQ water, dried overnight in an oven at 40 °C, and weighed with a microbalance (M2P; Sartorius). Bone marrow was extracted by cutting each bone lengthwise and physically removing interior material with a scalpel. After cleaning again with Milli-Q water, the bones from each individual were combined and ground with a cryogenic mill (CertiPrep 6750 Freezer/Mill, SPEX).

Whole body concentrations of Hg and Aroclor 1268 were determined for these hatchlings in a separated study (23). Analytical procedures used for the determination of whole body metals and Aroclor 1268 concentrations have been described in detail elsewhere (23, 25). Shortly, individuals were grounded using a cryogenic mill. A portion of the homogenized sample (100 mg) was used for metals determination and analyzed using inductively coupled plasma mass spectrometry (ICP–MS, Elan 6000, Perkin Elmer). In the remaining portion, PCBs were extracted using organic solvents and analyzed using a gas chromatograph equipped with an electron capture detector (GC 6890, Hewlett Packard). Identification of congeners was confirmed by mass spectrometric analyses.

The chemical composition of the combined leg bone was determined by ICP–MS and Fourier transform infrared spectrometry (FTIR). For the ICP–MS analyses, 3 mg of bone powder was microwave digested in a 10 mL solution of 10% HNO₃ and 3% H₂O₂. Ca and P concentrations were measured using an Elan 6000 ICP–MS (Perkin-Elmer). The precision of the chemical analyses was better than 1 ppm. For quality control purposes, standard trace grade solutions containing Ca and P in sample range concentrations were prepared and analyzed after every five samples. All standard solutions were within 5% of the reported values.

For the FTIR analyses, 3 mg of bone powder was mixed with 200 mg of FTIR-grade KBr and pressed under vacuum at 9 metric tons of pressure for 20 min. Infrared spectra were recorded using a FTIR spectrometer (Magna IR 860, Nicolet) at 2 cm⁻¹ resolution over 1024 scans. The amounts of phosphate, carbonate, and organic matrix in bone were determined from the peak area of absorption bands associated with phosphate, carbonate, and amide groups in the

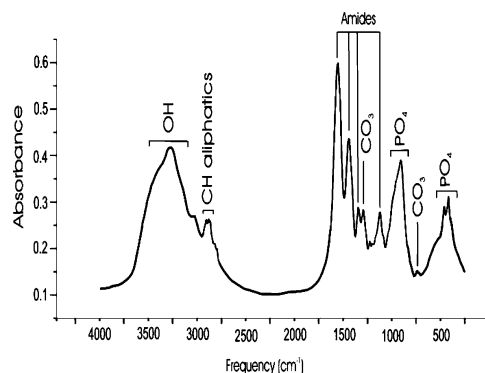


FIGURE 1. FTIR spectrum of a Clapper Rail chick bone. The peaks displayed in the spectrum correspond to the absorption of different molecular species present in bone tissue. The intensity of the peaks is proportional to the concentration of the component associated to the absorption band.

infrared spectra (Figure 1). In case of a single molecular group generating several bands, only peaks from the most intense band were used for analyses (e.g., peaks from phosphate band at 900–1200 cm⁻¹ were used rather than those from 500 to 650 cm⁻¹). Overlapping peaks were resolved and their integrated areas measured using curve fitting software (Peakfit). This software separated overlapping peaks using a second derivative methodology, allowing for a detailed and quantitative analysis of different molecular constituents of bone mineral. For this methodology, Peakfit software needs to set several parameters. Specifically, for our analyses, peaks were fitted to a mixed Gaussian + Lorentzian function. Peak amplitude and position were allowed to vary within 10% and 10 cm⁻¹, respectively, while curve shape and width were allowed to vary freely. The degree of smoothing was set at 10% (at the 1200–900 cm⁻¹ band) and 15% (at the 1950–1350 cm⁻¹ band). The number of peaks in each band analyzed was held constant. For the 1200–900 cm⁻¹ and the 1950–1350 cm⁻¹ bands, the number of peaks was seven and four, respectively. Locations of peaks in the two main regions analyzed are shown in Figure 2.

Even in carefully prepared KBr disks, sample powder is not homogeneously distributed resulting in large variation of absolute peak intensities in the FTIR spectra. To minimize this effect and be able to do quantitative comparison among samples, peak areas were normalized to the area of 3800–2800 cm⁻¹ band region associated with OH groups (Figure 1). The peak area of C–H stretching band was removed from this region. The resulting area ratios are represented by a capital “A”, followed by the peak or band position (e.g., A1660, A1405). Alternatively, to evaluate the contribution of individual components, associated to different molecular environments, within a particular region (e.g., HPO₄ within the PO₄ band region), deconvoluted peak areas were normalized to the relevant band region area from which they derived (e.g., A900_1200). These peak area ratios are designated using “a”, followed by the peak position (e.g., a953, a985, a1010). Error in the determination of normalized peak areas was 10% or less. This error was estimated by repeatedly measuring the same sample 10 times.

The following parameters were used to characterize bone composition and crystallinity from FTIR analyses:

The degree of mineralization of bone (mineral) (2) is defined as the mineral to organic matrix ratio and was estimated as follows:

$$\text{mineral} = A1200_900/A1660 \quad (1)$$

where A1200_900 represent the amount of phosphate in bone and A1660 the amount of amide I groups (main band from bone organic matrix).

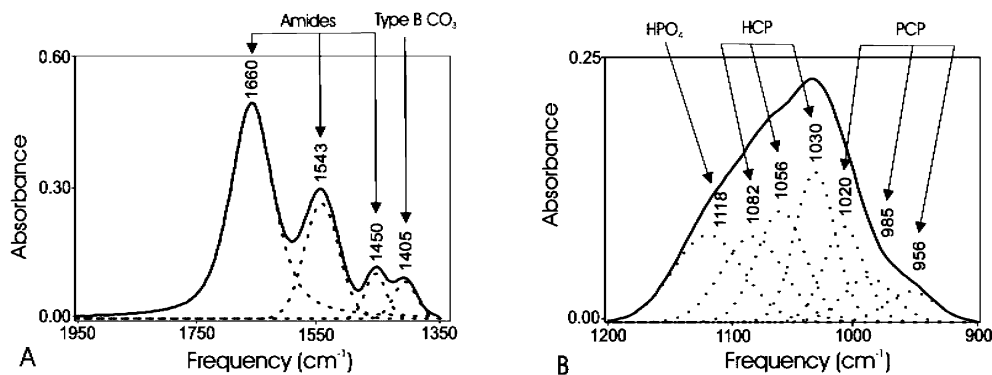


FIGURE 2. Overlapping peaks constituting two main band regions. (A) The 1950–1350 cm^{-1} region contains three main peaks, 1660, 1550, and 1450 cm^{-1} , associated with different vibration modes for amide groups in collagen, and a fourth peak at 1405 cm^{-1} for carbonate groups (type B mineral substitution). (B) The 1200–900 cm^{-1} band region, associated with phosphate groups, is composed of seven peaks. HCP and PCP stand for highly and poorly crystalline phosphate, respectively.

TABLE 1. Concentration of Toxicants (Hg and PCB mixture (Aroclor 1268)) Sediments, Fiddler Crabs, and Clapper Rail Hatchlings (whole body load), from Blythe Island and LCP Site^a

	Blythe Island		LCP site		ANOVA test			
	mean \pm SD	N	mean \pm SD	N	F	df1	df2	P
Sediments								
Hg (ppm)	0.18 \pm 0.07	10	2.3 \pm 1.1	15	65.31	1	23	<0.0001
PCB (ppm)	0.35 \pm 0.03	10	5 \pm 2	15	92.74	1	23	<0.0001
Crabs								
Hg (ppm)	0.14 \pm 0.01	10	0.43 \pm 0.05	15	86.13	1	23	<0.0001
PCB (ppm)	0.33 \pm 0.06	10	1.7 \pm 0.3	15	65.36	1	23	< 0.0001
Clapper Rail Hatchlings								
Hg (ppm)	1.8 \pm 1.2	15	4.6 \pm 1.6	16	30.01	1	26	< 0.001
PCB (ppm)	28 \pm 19	15	222 \pm 213	16	12.38	1	28	0.001

^a Concentrations are reported in dry weight. SD is the standard deviation; df is degrees of freedom.

The relative amount of carbonate in bone mineral (minCO3) was calculated as the ratio of the peak area for 1405 cm^{-1} (A1405; mainly carbonate type B substitution) to phosphate band area (A1200_900) and was calculated as follows:

$$\text{minCO3} = \text{A1405/A1200_900} \quad (2)$$

The crystallinity index (CI) (26) was calculated as the ratio between peak areas at 1030 cm^{-1} (highly crystalline apatite) to 1010 cm^{-1} (poorly crystalline apatite):

$$\text{CI} = \text{A1030/A1010} \quad (3)$$

Basic descriptive statistics were used to characterize bone properties. Analysis of variance (one-way ANOVA) was used to compare bone properties between the two populations to gain insights into the effects of contaminants on bone growth and metabolism. Pearson's correlation analysis and linear regression models were used to study relationships among the different properties of bone and the concentrations of toxicants in whole body analyses to determine how contaminants affect the chemical composition of bone. The level of significance chosen for all analyses was $P \leq 0.05$. Prior to these analyses, variables were tested for normality using Shapiro–Wilk statistics. All variables passed the normality test ($P > 0.05$) with the exception of Hg and PCB concentrations. After a log-transformation, these variables also had normal distributions. All statistical analyses were performed using the software package SPSS 12.0 (SPSS Inc.).

Results

Toxicants Levels at Blythe Island and LCP Site. Table 1 summarizes the toxicant levels reported in Cumbee (23) for

sediments, crabs (main food for Clapper Rail), and Clapper Rail hatchlings (used in this study) from the reference (Blythe Island) and contaminated site (LCP). These data show that Aroclor 1268 and Hg are at elevated levels in sediments and fauna in the contaminated marsh ecosystem, and at significantly higher concentrations than in the reference site. All contaminants found at LCP site were also detected at the reference site (though at significant lower levels), indicating that contaminants have migrated to nearby areas. Specifically, Hg and Aroclor 1268 levels in hatchlings are much higher than in sediments or food items, and comparable to those found in adult Clapper Rails (23). All these results show that toxicants are bioavailable and transferred from parents to offspring.

Bone Properties. Physical parameters and calculated peak areas are reported for leg bones in Table 2, along with the related ANOVA results. Neither the combined weight ($P = 0.891$) nor length ($P = 0.261$) of leg bones (femur + tibiae/fibulae) was significantly different between the two locations, however, important differences in the chemical composition of bones were detected. For instance, the Ca/P ratio was significantly greater in bones from the contaminated site than the reference site. Also, the relative amount of carbonate (minCO3) and acid phosphate (HPO_4 , estimated from a1118) in bone mineral were significantly lower in bones from the LCP site.

Correlation analyses for the chemical components of bone and whole body Hg and Aroclor 1268 concentrations are shown in Table 3. This information provides additional insights into processes that affect bone growth and metabolism. Concentrations of Hg and Aroclor 1268 were highly correlated indicating that these toxicants are associated. Additionally, some compositional parameters showed a

TABLE 2. Summary of Bone Property Values in Clapper Rail Hatchlings from Blythe Island and LCP Site

	Blythe Island		LCP site		ANOVA test			
	mean ± SD	N	mean ± SD	N	F	df1	df2	P
bone weight (g)	20 ± 7	20	20 ± 6	18	0.019	1	36	0.891
bone length (mm)	3.18 ± 0.33	19	3.02 ± 0.52	19	1.305	1	36	0.261
Ca/P	1.53 ± 0.05	16	1.58 ± 0.06	18	8.003	1	32	0.008
mineral	0.51 ± 0.11	20	0.55 ± 0.07	20	1.384	1	38	0.247
minCO3	0.13 ± 0.01	20	0.12 ± 0.01	20	6.208	1	38	0.017
crystallinity index	1.55 ± 0.20	20	1.54 ± 0.14	20	0.033	1	38	0.857
A1660	117 ± 9	20	117 ± 7	20	0.000	1	38	0.993
A1200_900	61 ± 12	20	65 ± 9	20	1.893	1	38	0.177
a953	0.06 ± 0.02	20	0.06 ± 0.01	20	0.321	1	38	0.574
a985	0.06 ± 0.02	20	0.06 ± 0.01	20	0.007	1	38	0.932
a1010	0.12 ± 0.01	20	0.12 ± 0.01	20	0.985	1	38	0.327
a1030	0.19 ± 0.02	20	0.19 ± 0.01	20	0.398	1	38	0.532
a1056	0.17 ± 0.01	20	0.18 ± 0.01	20	3.223	1	38	0.081
a1082	0.18 ± 0.01	20	0.18 ± 0.01	20	1.397	1	38	0.245
a1118	0.23 ± 0.03	20	0.22 ± 0.01	20	5.424	1	38	0.025

^a Values which were significantly different among two locations ($P < 0.05$) are highlighted in bold. SD is the standard deviation; df is degrees of freedom.

TABLE 3. Correlation Analyses for Different Bone Properties Determined in Clapper Rail Chicks and the Whole Body Load of Toxicants (Hg and PCB [Aroclor 1268]) in the Two Sites Studied^a

	Blythe Island						LCP site					
	log (Hg)			log (PCB)			log (Hg)			log (PCB)		
	R	P	N	R	P	N	R	P	N	R	P	N
log (Hg)	1						1					
log (PCB)	0.814	<0.001	15	1			0.717	0.002	16	1		
bone weight	-0.280	0.313	15	-0.321	0.244	15	0.037	0.900	14	0.347	0.224	14
bone length	-0.105	0.720	14	-0.160	0.584	14	0.514	0.050	15	0.409	0.130	15
Ca/P	0.303	0.338	12	0.321	0.309	12	-0.019	0.950	14	-0.081	0.784	14
mineral	0.273	0.325	15	0.175	0.534	15	0.139	0.608	16	0.175	0.517	16
minCO3	-0.403	0.137	15	-0.303	0.272	15	-0.133	0.624	16	-0.006	0.984	16
crystallinity index	0.334	0.223	15	0.305	0.269	15	0.548	0.028	16	0.697	0.003	16
A1660	-0.001	0.996	15	0.043	0.879	15	0.184	0.494	16	0.014	0.959	16
A1200_900	0.329	0.232	15	0.214	0.444	15	0.240	0.370	16	0.197	0.463	16
a953	0.359	0.189	15	0.420	0.119	15	-0.107	0.693	16	0.075	0.783	16
a985	-0.229	0.412	15	-0.208	0.457	15	-0.594	0.015	16	-0.731	0.001	16
a1010	-0.490	0.064	15	-0.418	0.121	15	-0.521	0.038	16	-0.699	0.003	16
a1030	0.056	0.843	15	0.070	0.805	15	0.349	0.185	16	0.397	0.128	16
a1056	0.316	0.251	15	0.226	0.417	15	0.655	0.006	16	0.711	0.002	16
a1082	0.132	0.640	15	-0.006	0.983	15	0.200	0.457	16	0.330	0.212	16
a1118	-0.197	0.482	15	-0.119	0.673	15	0.085	0.754	16	0.004	0.988	16

^a Properties and values of Pearson correlation coefficients (R) are at $P < 0.05$ are highlighted in bold. N is the number of observations.

significant correlation with toxicant levels at the contaminated site and none at the reference site. Specifically, at the contaminated site, the crystallinity index of bone was strongly and positively correlated to the Aroclor 1268 load of chicks ($R = 0.697$; $P < 0.003$; $N = 16$), indicating that exposure to Aroclor 1268 increased the crystallinity of bone mineral (Table 3; Figure 3a). This is in agreement with the positive correlation between some of the peak areas associated to highly crystalline phosphate (a1030, a1056) and Aroclor 1268 concentration and also with the negative correlation between the peak areas associated to poorly crystalline phosphate (a985 and a1010) and Aroclor 1268 concentration. Other changes related to contamination such as Ca/P ratio, minCO3, and HPO₄ content (a1118), which differed among sites, showed no statistically significant relationship to PCBs or Hg concentrations individually. Nevertheless, all observed changes and relationships are consistent and indicated that toxicants are inducing the formation of bone mineral with a composition characteristic of more mature bone (higher Ca/P, lower carbonate and acid phosphate content, increased crystallinity). The same relationships described for Aroclor

1268 were also observed for Hg for the same bone compositional parameters, though correlations with this metal were always lower than with Aroclor 1268 (Table 3). Additionally, a marginally significant correlation between bone length and Hg concentration was found at LCP site (Table 3). None of the previously described relationships were observed at the control site for either contaminant.

Discussion

This investigation is part of a larger ongoing study aimed to quantify the impact of contamination on the LCP superfund site using Clapper Rails as an indicator species of the health of this marsh ecosystem (23–25, 27). Previous studies, focused on Clapper Rail adults and hatchlings from LCP, indicated that toxicants present in the area have affected individuals at different levels compromising genetic (high incidence of DNA strand breakage in adults), reproductive (eggshell thinning), and hatchling (inability to stand, limb defects) health (23–25, 27). The current study on bone characteristics gives additional information on the effect of toxicants present at LCP on the overall health of Clapper Rail populations.

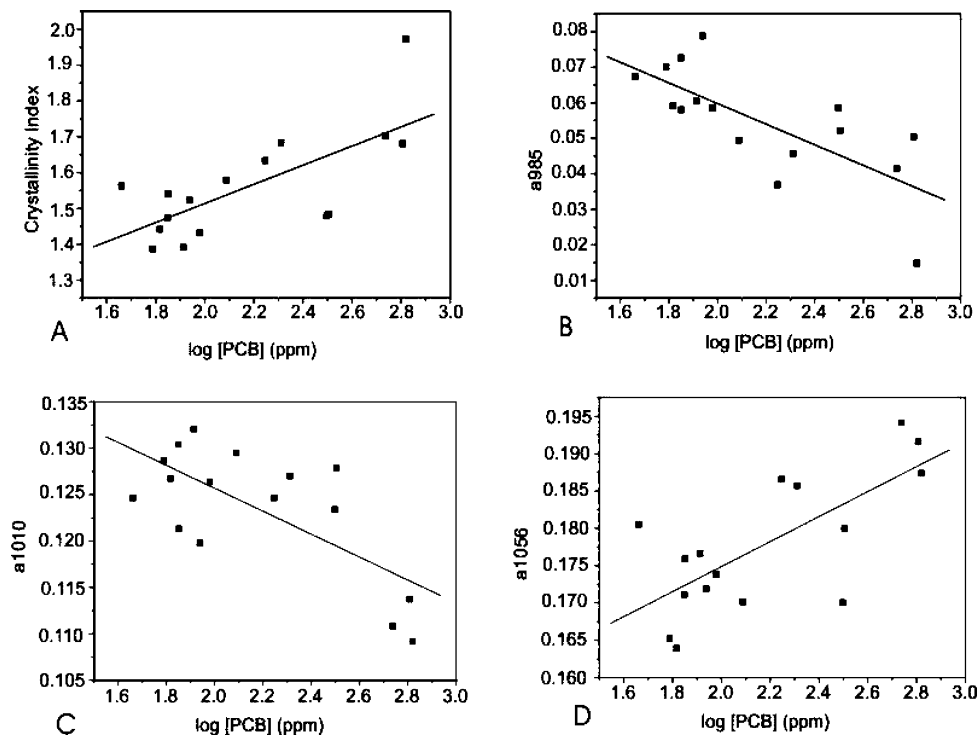


FIGURE 3. Relationships between bone composition and contaminant levels at the LCP site. (A) Crystallinity of bone mineral, estimated as the crystallinity index, increases with PCBs (Aroclor 1268) load. (B) Area of peak at 985 cm^{-1} associated with poorly crystalline phosphate decreases as PCBs load increases. (C) Area of peak at 1010 cm^{-1} associated with poorly crystalline phosphate decreases as PCBs load increases. (D) Area of peak at 1056 cm^{-1} associated with highly crystalline phosphate increases with PCBs load.

The main contaminants in the LCP marsh system, Aroclor 1268 and Hg, were at elevated levels in Clapper Rail hatchlings, well above the action levels of 2 and 1 ppm defined by the U.S. Food and Drug Administration (FDA) for PCBs and Hg, respectively, in fish as a food commodity (28, 29). Toxicant levels found in biota at LCP area have evolved over time. Reported levels of Hg for crabs were 0.94 ppm in 1971, 0.7 ppm in 1987, and 0.43 ppm in 2002 (16, 23). Hg levels for Clapper Rails in 1971 ranged from 2.0 to 9.5 ppm compared to 1.25 ppm in 1995, and 1.4 ppm in 2002, in muscle tissue (wet weight) (16, 19, 23). Reported values for PCBs in Clapper rail captured at LCP in 1995 were 24.5 ppm, and individuals captured in 2002 were 16 ppm in muscle tissue (wet weight) (19, 23). Thus, it seems that both Hg and PCBs levels have decreased overtime but levels are still very high. On the other hand, large variations in toxicant levels found in Clapper Rail hatchlings, especially for Aroclor 1268 at LCP, are probably due to spatially heterogeneous distribution of toxicants (17, 19, 23). In fact, Cumbee (23) showed that the adult Clapper Rails from LCP with the highest Aroclor 1268 levels also had the highest soil Aroclor 1268 levels in their home ranges. Cumbee's home range study was specifically designed to examine this potential phenomenon; however, it should be noted that the eggs collected for this study were not associated with those particular adults to avoid changing female behavior. Regardless, the sample size in that study is robust enough to infer that this would also be the case for the chicks used in this study.

The reference location (Blythe Island) was chosen to determine how ambient levels in the environment compare to those of the perturbed site (LCP site) and if contamination has affected the later. The reference location is representative of the biogeochemistry and physical geography of the focal site, but with toxicants being studied at background levels. One difficulty in choosing a reference site in any estuarine ecosystem is that is almost impossible to avoid all potential toxicants. In fact all the contaminants found at LCP were also detected at lower levels at the reference site and

throughout the estuaries in Brunswick (23), most likely transported through movement of silt and sediment from tidal flows. Congener formulation of Aroclor 1268 is quite uncommon, and its presence in the reference site can only be attributed as originating from LCP and can be considered as a marker of origin. The fact that contaminants found at LCP were also detected within the entire Brunswick estuary at lower levels supports the hypothesis that observed deleterious effects to our focal endpoints at the contaminated site were caused by the elevated levels of these contaminants.

Regarding the described effects on bone, it is difficult to know which specific toxicant(s) induced them, especially when they are associated (Hg and PCB co-varied) as in this study. Moreover, toxicant mixtures (e.g., toxicants that were not elevated in biota but present in the LCP system) may influence biokinetics. For instance, other organochlorines (e.g., dioxins) has been detected at significant levels at LCP system (30). Heavy metals, including Hg, are known to disrupt condrocyte metabolism reducing long bone development (11). Also, Hg affects calcium metabolism (4) and exposure to Hg may be manifested in bone mineral loss and osteoporosis or osteomalacia (8). Even though Clapper Rail hatchlings in the LCP system accumulated relatively high levels of Hg, we did not find any difference in length or degree of mineralization of leg bone compared to the control group. Since we did not observe these types of changes, we deduced that Hg levels could be too low to induce these effects. On the other hand, the main detectable effects of contamination on bone were an alteration of its chemical composition and crystallinity. Specifically, bone in the contaminated site had a higher Ca/P ratio, and lower carbonate and acid phosphate content. As described in the Introduction, these compositional changes are typically associated to bone maturation. Additionally, FTIR spectrometry data revealed a dose dependent change in the crystallinity of bone mineral which increased with toxicant levels. This is further confirmed by the observed increase of proportion of highly crystalline phosphate relative to poorly crystalline with toxicant levels.

All these observations indicated that contamination has accelerated bone maturation in Clapper Rail at LCP site. This could be due to a reduction in the rate of bone turnover caused by contaminants resulting in a higher amount of mature bone mineral relative to newer bone mineral. Also, a reduction in bone remodeling would reduce the availability of Ca for eggshell formation explaining the eggshell thinning observed in previous studies (24). Other authors (9, 10) have also associated an alteration of bone remodeling to PCBs exposure. Based on similarity of effects reported for PCBs and stronger correlations of bone compositional parameters with Aroclor 1268 loads, the observed changes can be attributed to a hormonal alteration of bone metabolism induced by exposure to PCBs. In any case, the altered composition and crystallinity is indicative of a pathological condition of bone (1, 2). Furthermore, to our knowledge, PCBs levels found in hatchling at LCP are higher than any reported value in eggs collected in other contaminated sites and well above levels for observable effects in laboratory tests in chicken embryos (14). Regardless, we cannot ignore the possibility of a cooperative effect of both Aroclor 1268 and Hg as well as other toxicants (e.g., dioxins) present at lower levels, although exploratory data analysis did not reveal any such associations.

In conclusion, these results indicate that bone in chicks from the contaminated site has matured more rapidly than in chicks from the reference site. This could be due to a reduction in the rate of bone turnover caused by an alteration of bone metabolism most probably caused by Aroclor 1268 or through a cooperative effect with Hg. Finally, in future studies, we will perform controlled dose experiments using Aroclor 1268 and/or Hg to further study the influence of these contaminants on bone metabolism. Also, the same methodology will be applied to quantify the environmental impacts of these or other contaminants on other ecosystems. Last, although this toxicological endpoint must come from sacrificed individuals, in cases such as those at LCP where chicks have toxicological abnormalities, it can advance the understanding of the evolutionary trajectory of effected populations and lend insight into the broader question of whether such systems are ecological traps.

Acknowledgments

This study has been financed by an award (DE-FC09-96-SR18546) from the U.S. Department of Energy to the University of Georgia, Savannah River Ecology Laboratory (SREL). We also thank financial support from the Spanish government through grant REN2003-07375 and Programa Ramon y Cajal. We thank Brian Jackson (SREL) for the chemical analysis of samples by ICP-MS, Jason Enelow for help with FTIR analyses, and James C. Cumbee Jr., and Warren L. Stephens, Jr. for egg collection and incubation. Finally, we would like to thank three anonymous referees for their useful comments and corrections.

Literature Cited

- Glimcher, M. J. (The nature of the mineral phase in bone: Biological and clinical implications. In *Metabolic Bone Disease and Clinically Related Disorders*; Alvioli, L. V., Krane, S. M., Eds.; Academic Press: San Diego, CA, 1998; pp 23–50.
- Boskey, A.; Mendelsohn, R. Infrared analysis of bone in health and disease. *J. Biomed. Opt.* **2005**, *10*, 031102-1–031102-9.
- Baxley, M. N.; Hood, R. D.; Vedel, G. C.; Harrison, W. P.; Szczech, G. M. Prenatal toxicity of orally administered sodium arsenite in mice. *Bull. Environ. Contam. Toxicol.* **1981**, *26*, 749–756.
- Scheuhammer, A. M. The chronic toxicity of aluminium, cadmium, mercury, and lead in birds: A review. *Environ. Pollut.* **1987**, *46*, 263–295.
- Kanti, A.; Smith, M. A. Effects of heavy metals on chondrogenic differentiation of embryonic chick limb cells. *Toxicol. in Vitro* **1997**, *10*, 329–338.

- Andrews, J. E. Polychlorinated biphenyl (Arochlor 1254) induced changes in femur morphometry calcium metabolism and nephrotoxicity. *Toxicology* **1989**, *57*, 83–96.
- Lind, P. M.; Eriksen, E. F.; Sahlin, L.; Edlund, M.; Örborg, J. Effects of the Antiestrogenic Environmental Pollutant 3,3',4,4',5-Pentachlorobiphenyl (PCB #126) in Rat Bone and Uterus: Diverging Effects in Ovariectomized and Intact Animals. *Toxicol. Appl. Pharm.* **1999**, *154*, 236–244.
- Jin, G. B.; Inoue, S.; Urano, T.; Cho, S.; Ouchi, Y.; Cyong, J. C. Induction of anti-metallothionein antibody and mercury treatment decreases bone mineral density in mice. *Toxicol. Appl. Pharm.* **2002**, *185*, 98–110.
- Lee, L. L.; Lee, J. S. C.; Waldman, S. D.; Casper, R. F.; Grynepas, M. D. Polycyclic aromatic hydrocarbons present in cigarette smoke cause bone loss in an ovariectomized rat model. *Bone* **2002**, *30*, 917–923.
- Lind, P. M.; Milnes, M. R.; Lundberg, R.; Bermudez, D.; Orberg, J.; Guillette, L. J., Jr. Abnormal bone composition in female juvenile American alligators from a pesticide-polluted lake (Lake Apopka, Florida). *Environ. Health Perspect.* **2004**, *112*, 359–362.
- Puzas, J. E.; Sichel, M. J.; Felter, M. E. Osteoblasts and chondrocytes are important target cells for the toxic effects of lead. *Neurotoxicology* **1992**, *13*, 783–788.
- Singh, S. U. N.; Casper, R. F.; Fritz, P. C.; Sukhu, B.; Ganss, B.; Girard, B., Jr.; Savouret, J. F.; Tenenbaum, H. C. Inhibition of dioxin effects on bone formation in vitro by a newly described aryl hydrocarbon receptor antagonist, resveratrol. *J. Endocrinol.* **2000**, *167*, 183–195.
- Sonne, C.; Dietz, R.; Born, E. W.; Riget, F. F.; Kirkegaard, M.; Hyldstrup, L.; Letcher, R. J.; Muir, D. C. G. Is bone mineral composition disrupted by organochlorines in East Greenland polar bears (*Ursus maritimus*)?. *Environ. Health Perspect.* **2004**, *112*, 1711–1716.
- Gould, J. C.; Cooper, K. R.; Scanes, C. G. Effect of polychlorinated biphenyls mixtures and three specific congeners on growth and circulating growth-related hormones. *Gen. Comput. Endocrinol.* **1997**, *106*, 221–230.
- Beland, P.; DeGuise, S.; Girard, C.; Lagace, A.; Martineau, D.; Michaud, R.; Muir, D. C. G.; Norstrom, R. J.; Pelletier, E.; Ray, S.; Shugart, L. R. Toxic compounds and health and reproductive effects in St. Lawrence beluga whales. *J. Great Lakes Res.* **1993**, *19*, 766–775.
- U.S. EPA. *Site Inspection Report, LCP Chemicals-Georgia Inc., Brunswick, Glynn County, Georgia*; United States Environmental Protection Agency, Waste Management Division: Washington, DC, 1990.
- Kannan, K.; Maruya, K. A.; Tanabe, S. Distribution and characterization of polychlorinated biphenyl congeners in soil and sediments from a superfund site contaminated with Aroclor 1268. *Environ. Sci. Technol.* **1997**, *31*, 1483–1488.
- Kannan, K.; Nakata, H.; Stafford, R.; Masson, G. R.; Tanabe, S.; Giesy, J. P. Bioaccumulation and toxic potential of extremely hydrophobic polychlorinated biphenyl congeners in biota collected at a superfund site contaminated with Aroclor 1268. *Environ. Sci. Technol.* **1998**, *32*, 1214–1221.
- Sprenger, M. D.; Finley, N. J.; Huston, M. *Ecological assessment, ecological risk evaluation of the salt marsh and adjacent areas at the LCP superfund site, Brunswick GA*, MAS 0000300; U.S. Environmental Protection Agency: Washington, DC, 1997.
- Gaines, K. F.; Cumbee, J. C., Jr.; Stephens, W. L., Jr. Nest characteristics of the clapper rail in coastal Georgia. *J. Field Ornithol.* **2003**, *74*, 152–156.
- Terres, J. K. *The Audubon Society Encyclopedia of North American Birds*; Wings Books: NJ, 1991; p 1109.
- Eddleman, W. R.; Conway, C. J. Clapper Rail (*Rallus longirostris*). In *The birds of North America*, no. 340; Poole, A.; Gill, F., Eds.; The Birds of North America, Inc.: Philadelphia, PA, 1998.
- Cumbee, J. C., Jr. Quantifying trophic transfer of mercury and Aroclor 1268 to clapper rails (*Rallus longirostris*) in a contaminated ecosystem using spatially explicit sampling methods. MSc Thesis, The University of Georgia, 2003.
- Rodriguez-Navarro, A. B.; Gaines, K. F.; Romanek, C. S.; Masson, G. R. Mineralization of Clapper Rail eggshell from a contaminated salt marsh system. *Arch. Environ. Contam. Toxicol.* **2002**, *43*, 449–460.
- Novak, J. M.; Gaines, K. F.; Cumbee, J. C., Jr.; Mills, G. L.; Rodriguez-Navarro, A.; Romanek, C. S. Clapper Rails as Indicator Species of Estuarine Marsh Health. *Stud. Avian Biol.* (in press).
- Miller, L. M.; Vairavamurthy, V.; Chance, M. R.; Mendelsohn, R.; Paschalis, E. P.; Betts, F.; Boskey, A. L. In situ analysis of mineral content and crystallinity in bone using infrared micro-

- spectroscopy of the ν_4 PO₄⁻ vibration. *Biochim. Biophys. Acta* **2001**, 1527, 11–19.
- (27) Gaines, K. F.; Novak, J. M.; Stephens, W. L., Jr.; Cumbee, J. C., Jr. Determination of Contaminant Burdens, DNA Strand Breakage and Nesting Success in Clapper Rails Inhabiting the Salt Marsh Estuary in Brunswick, GA—PART I & II. In *Report to the U.S. Fish and Wildlife Service*; U.S. Fish and Wildlife Service: U.S. Government Printing Office: Washington, DC, 2000.
- (28) Tolerances for polychlorinated biphenyls (PCB's). *Code of Federal Regulations*, Section 30, Part 109, 1998.
- (29) *Fish, Shellfish, Crustaceans and Other Aquatic Animals—Fresh, Frozen, or Processed—Methyl Mercury*; (CPG 7108.07). Sec. 540.600 (rev 3/95). Compliance Policy Guides. Department of Health and Human Services, Food and Drug Administration: Washington, DC, 1996.
- (30) Kannan, K.; Watanabe, S.; Giesy, J. P. Congener profile of polychlorinated/brominated dibenzo-*p*-dioxins and dibenzofurans in soil and sediments collected at a former chlor-alkali plant. *Toxicol. Environ. Chem.* **1998**, 67, 135–167.

Received for review March 31, 2006. Revised manuscript received June 13, 2006. Accepted June 13, 2006.

ES060769X