January 2006

The Clapper Rail as an Indicator Species of Estuarine Marsh Health

James N. Novak
Eastern Illinois University

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

James C. Cumbee Jr.
University of Georgia

Gary L. Mills
University of Georgia

Alejandro Rodriguez-Navarro

See next page for additional authors

Studies in Avian Biology No. 32:270–281

THE CLAPPER RAIL AS AN INDICATOR SPECIES OF ESTUARINE-MARSH HEALTH

JAMES M. NOVAK, KAREN F. GAINES, JAMES C. CUMBEE, JR., GARY L. MILLS, ALEJANDRO RODRIGUEZ-NAVARRO, AND CHRISTOPHER S. ROMANEK

Abstract. Clapper Rails (*Rallus longirostris*) can potentially serve as an indicator species of estuarine-marsh health because of their strong site fidelity and predictable diet consisting predominantly of benthic organisms. These feeding habits increase the likelihood of individuals accumulating significant amounts of contaminants associated with coastal sediments. Moreover, since Clapper Rails are threatened in most of their western range, additional study of the effects of potential toxins on these birds is essential to conservation programs for this species. Here we present techniques (DNA strand breakage, eggshell structure, and human-consumption risk) that can be used to quantify detrimental effects to Clapper Rails exposed to multiple contaminants in disturbed ecosystems as well as humans who may eat them. Adult birds collected near a site contaminated with polychlorinated biphenyls (PCBs) and metals in Brunswick, Georgia had a high degree of strand breakage, while those collected from a nearby reference area had no strand breakage. Although, results showed that eggshell integrity was compromised in eggs from the contaminated sites, these results were more diffuse, re-emphasizing that multiple endpoints should be used in ecological assessments. This study also shows that techniques such as eggshell integrity on hatched eggs and DNA strand breakage in adults can be used as non-lethal mechanisms to monitor the population health of more threatened populations such as those in the western US. We also present results from human-based risk assessment for PCBs as a third toxicological endpoint, since these species are hunted and consumed by the public in the southeastern US. Using standard human-risk thresholds, we show a potential risk to hunters who consume Clapper Rails shot near the contaminated site from PCBs because of the additional lifetime cancer risk associated with that consumption.

Key Words: Clapper Rail, DNA strand breakage, eggshell integrity, indicator species, metals, polychlorinated biphenyl, *Rallus longirostris.*

EL RASCÓN PICUDO COMO ESPECIE INDICADORA DE LA SALUD DE MARISMAS ESTUARINOS.

Resumen. Los Rascones Picudos (*Rallus longirostris*) pueden servir potencialmente como una especie indicadora de la salud de marismas estuarinos, gracias a su fuerte fidelidad al sitio y a su predecible dieta que consiste predominantemente en organismos bentónicos. Estos hábitos alimenticios incrementan la posibilidad de individuos que acumulan cantidades significativas de contaminantes asociados con sedimentos costeros. Además, ya que los Rascones Picudos se encuentran en peligro en casi todo su rango oeste, estudios adicionales de los efectos de toxinas potenciales en estas aves es esencial para los programas de conservación para estas especies. Aquí presentamos técnicas (rompimiento de ADN, estructura de cáscara de huevo, y riesgo de consumo humano) que pueden ser utilizadas para cuantificar efectos detrimentales para los Rascones Picudos, expuestas a múltiples contaminantes en ecosistemas en disturbio, como también en humanos que los consumen. Las aves adultas colectadas cerca de un sitio contaminado con bifenil policlorinatado (BPC) y metales en Brunswick, Georgia tienen un alto grado de rompimiento de ADN, mientras que aquellos colectados de un área de referencia cercana no tenían rompimiento de ADN. A pesar de que los resultados muestran que la integridad de la cáscara de huevo estuvo comprometida en huevos del agua contaminada, estos resultados fueron más difusos, re-enfatizando que múltiples puntos finales deberían ser utilizados en valoraciones ecológicas. Este estudio también muestra que técnicas tales como integridad de cáscara de huevo en huevos eclosionados y rompimiento de ADN en adultos pueden ser utilizados como mecanismos no letales, para el monitoreo de la salud de la población de más poblaciones en peligro, tales como aquellas en el oeste de EU. También presentamos resultados de valoración del riesgo basado en el humano para BPC como un punto final toxicológico tercero, ya que estas especies son cazadas y consumidas por el público en el sureste de EU. Utilizando umbrales estándar de riesgo humano, mostramos un potencial riesgo para los cazadores que consumen Rascones Picudos matadas cerca de sitios contaminados por BPC, debido al riesgo de cáncer adicional de toda la vida, asociado con ese consumo.

Saltmarsh habitats along the Atlantic and Pacific coasts are biologically and economically valuable natural resource areas. These areas not only attract tourists for recreation, but the abundance of the marsh's seemingly unending resources have lured industries to capitalize on the easy access to the open ocean's busy shipping lanes. Consequently, it becomes increasingly important to protect these fragile ecosystems from the effects of pollution and other anthropogenic disturbances. Since it is not practical to monitor every potential response to environmental impacts, studies must choose appropriate endpoints. In estuarine systems, wildlife can be extremely useful as indicators of the overall health of associated marshlands, especially to address the consequences of ecotoxicological disturbances. In the case of environmental pollution in saltmarsh systems, disturbances can have effects at multiple spatial scales. For example, due to their geochemical properties and mode of introduction into the environment, pollutants can often be studied at the local scale (hectares), whereas others tend to spread to the landscape scale requiring a spatial extent of many square kilometers (Hooper et al 1991, Crimmins et al. 2002). Therefore, to address concerns that may be approached at multiple scales, the proper species must be utilized to indicate if there are deleterious effects. In such cases, birds, specifically rails (Rallidae), are excellent species to monitor since they utilize these systems at both the local and landscape level. Further, genotoxicological and reproductive endpoints can be used to quantify and better understand the long-term effects that these disturbances may have to an estuary.

The Clapper Rail (*Rallus longirostris*) is a secretive marsh bird found throughout coastal saltmarshes from the Gulf of Mexico to Rhode Island and along California's Pacific coastline. The rail's strong site fidelity (Zembal et al. 1989) and predictable diet (Terres 1991) makes it an ideal organism to study the movement and fate of contaminants in disturbed ecosystems. In addition, this species is an integral part of the saltmarsh ecosystem, feeds relatively high on the food chain, is abundant throughout the East Coast, and is a popular game species in the Southeast. Conversely, the Pacific coastal populations are threatened due to habitat destruction and pollution (Eddleman and Conway 1998) and are thus not as amenable to study and experimentation. Using the Clapper Rail as an indicator species not only provides a way of assessing ecosystem health, but information of the relative toxicant burdens can be used to inform the public about potential health risks in areas where they may fish or hunt. Further, since rails are hunted, consuming birds that have inhabited contaminated areas may also present a direct risk to humans.

In coastal Georgia, large expanses of saltmarsh have abundant populations of Clapper Rails throughout the year. In the coastal city of Brunswick, Georgia, with its proximity to major shipping lanes, these marshes are host to many industries making them susceptible to industrial contamination. For example, a chlor-alkali plant discharged as much as 1 kg of mercury (Hg) a day for a period of 6 yr ending in 1972 in this region (Gardner et al. 1978). This site still has elevated levels of Hg as well as the polychlorinated biphenyl (PCB) Aroclor 1268 and other contaminants as will be shown in this paper. The effects of these pollutants have been of concern for many years to the residents of Brunswick as well as to government agencies such as the Environmental Protection Agency (EPA) and USDI Fish and Wildlife Service. Therefore, using Clapper Rails as indicator species in this estuary can address toxicant issues in the Brunswick area, as well as similarly impacted East Coast populations, and can contribute to information needed for the management of endangered subspecies such as the Light-footed Clapper Rail populations in California estuaries where individuals cannot be studied as intensely as in this investigation (Lonzarich et al. 1992).

Contaminant loads for rails, their food items, and their habitat have been established for the endangered California populations but only one study has been conducted in Brunswick, Georgia (Gardner et al. 1978, Lonzarich et al. 1992). San Francisco Bay has had a history of contamination of PCB's since the 1950s. Eggs from the Light-footed Clapper Rail were found to have elevated levels of PCBs as well as selenium (Se) and Hg. However, the effects of these toxicants could not be pursued any further because of limitations of sampling methodology and the rails endangered status (Lonzarich et al. 1992). Therefore, little information exists concerning lethal and sublethal effects that may have occurred or are occurring due to toxicant exposure which could have serious implications for the recovery of this species. We present the results of a study performed in Brunswick that will provide an example of how this species was used to address toxicological issues at the local scale by looking at reproductive effects (eggshell integrity), and at the landscape level by looking at genotoxicological effects to the rails themselves (DNA strand breakage). Finally, we determine what the probability of humans developing cancer would be from consuming Clapper Rail flesh (additional lifetime cancer

risk defined as the probability of contracting cancer over the individual's lifetime compared to the expected probability of contracting cancer if the individual had no contaminant exposure) from a marsh located next to a chemical plant that released PCBs and metals as well as other marshes located a few kilometers away from the chemical plant to provide an toxicological endpoint that addresses human risk.

ECOLOGICAL ENDPOINTS

DNA Strand Breakage

Contaminants can interact directly and indirectly with DNA to cause damage. One of the most obvious genotoxic interactions of contaminants with DNA is the induction of DNA strand breaks. DNA strand breaks are among the most easily detected and quantified types of DNA damage (Theodorakis et al. 1994, Sugg et al. 1995). A variety of metal species (including Cr [VI], Ni [II], Co [II], Fe [III], Cd [II], and Pb [II]) are known to induce DNA strand breaks (Hartwig 1995), therefore this technique is extremely useful as an endpoint to quantify damage from toxicant effects. Further, increased DNA strand breakage within living cells has been correlated with PAHs, PCBs, and heavy metals (Theodorakis et al. 1994, Sugg et al. 1995, Siu et al. 2003). In addition, contaminants such as Hg are known to interfere with DNA repair mechanisms (Snyder and Lachman 1989) and indeed Sugg et al. (1995) found that the synergistic effects of Hg and ^{137}Cs on strand breakage were greater than the effects of each contaminant alone. Thus, strand break assays represent a useful endpoint for assessing the consequences of exposure to a mixture of contaminants.

Eggshell Integrity

The eggshell protects the developing embryo against both mechanical impacts and bacterial invasions. Further, it controls the exchange of water and gases through the pores, is the main calcium reservoir for skeletal formation, and supplies some of the magnesium required during embryogenesis (Richards and Packard 1996, Nys et al. 1999). Therefore, it is of fundamental importance for an adequate development of the embryo to ensure the quality and integrity of the eggshell. DDTs and other organochlorides (e.g., PCBs) can affect enzyme activity involved in calcium transportation and consequently eggshell thickness (Cooke 1973, Baird 1995). Further, trace-metal contaminants can also influence the mineralization of eggshell. Specifically, they can reduce the availability of calcium in the diet, interfere with calcium metabolism, and can interfere with the mineralization process itself by affecting the precipitation rate, mineralogy, size, and morphology of crystals that make up the eggshell (Rodriguez-Navarro et al. 2002b). Therefore, exploring eggshell mineralization and thickness can help to better understand how these co-contaminants may affect eggshell integrity, which provides an ecological endpoint for reproductive effects.

Lifetime Human Cancer Risk

In the Southeast and especially in the Brunswick area, Clapper Rails are a popular game species with hunters often achieving their bag limits (K. Giovengo, pers comm.). This was evident to the authors during our collections because many local residents asked us if we would give them the rail carcasses when we came back to the boat landing. Interestingly, after informing them that these birds were collected near an area contaminated with PCBs, some individuals were still interested in consuming the birds. Therefore, since the birds are hunted and consumed by humans, using additional lifetime cancer risk to humans from consuming Clapper Rails contaminated with PCBs in this region is an extremely appropriate endpoint. Studies in humans provide supportive evidence for potential carcinogenic and non-carcinogenic effects of PCBs (Environmental Protection Agency 1996). Further, the EPA provides a framework to evaluate risk to humans who may consume PCBs in their food items. This quantification takes into account the amount of PCB's in the muscle tissue of the food items, the ingestion rate, exposure rate, the body weight, and the expected lifetime of the exposed individual (Environmental Protection Agency 1992).

METHODS

Study Area

This study was conducted in the estuarine marshes near Brunswick, Glynn County, Georgia. Clapper Rails were collected to compare ecotoxicological data from a saltmarsh contaminated with PCBs and metals near a contaminated high-priority Superfund Site—Linden Chemicals and Plastics (LCP)—to other similar saltmarsh locations located a few kilometers away from LCP that were not directly contaminated from point sources. The contaminated LCP site is a saltmarsh system located on the western shore of the Brunswick peninsula and is similar in vegetation structure

to nearby saltmarsh systems. It has been classified as a Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA; also known as Superfund Site) by the EPA, primarily due to contamination by Hg and PCBs (Aroclor 1268). Both contaminants are present in elevated levels in the sediments and resident fauna (fiddler crabs [*Uca* spp.]) of this marsh (J. M. Novak et al., unpubl. data). Further, this specific Aroclor has not been produced by any other company on the East Coast of the US. Therefore, it can be used as a marker indicating that animals with measurable levels of this contaminant accumulated it because of its release from the LCP site. The reference marsh areas, Troupe Creek, Mackay River, and Blythe Island are all located near Brunswick. These sites were chosen as representative of the surrounding areas—having a similar vegetation profile, tidal influence, tidal-creek diversity, and water chemistry (Gaines et al. 2003). The vegetation and habitat structure of these marshes are consistent with most other southeastern saltmarshes, consisting primarily of cordgrass (*Spartina* spp.) interspersed with small patches of rushes (*Juncus* spp.) and intersected by tidal creeks.

Collection Techniques

Adult Clapper Rails (N = 30) were collected from November–December 1999 from three locations in the saltmarsh estuary in Brunswick: the LCP marsh (N = 10), the Mackay River (N = 8), and Troupe Creek (N = 12). Birds were collected in the field during the full-moon high tide, using a shotgun. Upon collection, blood was immediately taken from the bird by opening up the chest cavity and puncturing the heart and/or major blood vessels. Only three–five drops of blood were collected and stored in STE buffer (see strand breakage methods below) for DNA strand-breakage analyses and frozen in liquid nitrogen. The bird was then placed in a cooler and transported to the Savannah River Ecology Laboratory (SREL) immediately following the collection period. In the laboratory, birds were either immediately dissected for muscle and liver tissue or placed in a refrigerator and dissected the following day. These samples were stored in a standard scintillation vial and stored in a -20 C freezer until PCB and metal analysis (see methods below). All blood samples were immediately stored in an ultracold freezer. During the dissection process, birds were aged by bursa and plumage examination, sexed, and weighed.

Clapper Rail nest searches were performed from 15 March—June 2000 in the Blythe Island and the LCP marshes. If a nest was found with four or more eggs, the eggs were removed from the nest and brought back to SREL within 4 hr. If nests had <four eggs, they were revisited within a few days to collect a larger clutch. The width, length, and weight of each egg were measured and, on return to the laboratory, eggs were immediately put into an incubator. All eggs were incubated at 37.2 C at 87% relative humidity and rotated automatically every 12 hr. Eggshells from each clutch were saved for mineralization analyses. Eggs were monitored on a daily basis and detailed notes were taken when pipping was initiated. The number of eggs hatched, total incubation time, and pipping activity (including the number of eggs not hatched but pipped) were quantified for each clutch. After hatching was complete (determined by the chick being fully out of its shell for at least 12 hr) hatchlings were weighed, euthanized by cervical displacement, and then frozen for further analysis.

Eggshell Mineralization

Eggshell mineralization and thickness were quantified as described in Rodriguez-Navarro et al. (2002a). In brief, the mineral composition of eggshell was determined using a powder X-ray diffractometer (Scintag X1). A diffractogram was collected from a sample of ground shell to be used as a reference pattern for crystals having a completely random orientation (I_o). The structure can be characterized by measuring the area of peaks, calculating the area of the peak divided by a ground shell reference (I/I_o) and plotting the ratios against the angle for the normal of each peak compared to a reference plane. The breadth of this distribution at half the maximum height (e.g., at $I/I_o = 0.5$) is called full width at half maximum (FWHM). FWHM is used as a gauge of crystal orientation in a composite structure. The smaller the number of peaks and the narrower the FWHM distribution, the higher is the degree of crystal orientation in the shell. It is preferable for the crystal orientation of the shell to be low. That is, as the crystal orientation increases, the eggshell integrity will decrease and the shell will become weak. These weaknesses may be offset by the thickness of the eggshell. Therefore, the thickness of the shell was also measured at four points separated by 90º at the egg waist using a micrometer.

DNA Strand Breakage

Basic strand-breakage protocols were modified from Theodorakis et al. (1994) with modifications as listed below. Red blood cells were

collected from each Clapper Rail taken in the field. Blood samples were stored in STE buffer (100 mM NaCl, 100 mM Tris pH 8.0, 100 mM EDTA) until they were prepared for electrophoresis. Four separate plugs were made from each blood sample. This provided replication of individuals within gels (replications 1 and 2) and between gels (replications 3 and 4). Instead of using standard agarose gel electrophoresis, we used a pulsed-field agarose gel electrophoresis assay similar to the system described by Blocher et al. (1989) to measure double-strand breakage of DNA with the following modifications: (1) red blood cells were used as the source of intact nuclei, (2) a BioRad CHEF DR III system was used for pulsed-field agarose gel electrophoresis, (3) DNA was stained with Sybr Gold™ after electrophoresis, and (4) Samples were loaded into every other lane such that every sample was flanked by a negative control lane and three lanes were loaded with three different DNA size ladders to serve as positive controls. The specific run conditions were: run time of 16 hr, electrical potential of 2.5V/cm, pulse angle of 120°, angle change ramped from 40–120 sec over the run, 2.2 l of 0.5× TBE buffer cooled to 14 C and pumped at approximately 0.8 1/min. A commercial gel image analysis system (Eagle Eye II, Stratagene, La Jolla, CA) was used to capture images of the flourescence from the UV-illuminated DNA. Each gel was imaged using 17 different exposures for fine scale quantification of the amount of DNA damage.

Additional Lifetime Human Cancer Risk

The additional lifetime cancer risk from ingesting food items contaminated with PCBs is determined using a tiered approach from existing information provided by the EPA (1996). Specifically, slope factors are derived from linear extrapolation of dose response studies. This slope factor is multiplied by lifetime average exposure levels to estimate the risk of cancer. These calculations are based on generalized studies and therefore are not specific to individual Aroclors (Environmental Protection Agency 1996). The specific calculations used were

$$LADD = \frac{C \times IR \times ED}{(BW \times LT)} \quad \text{Equation 1}$$

$$Risk = LADD \times Slope \quad \text{Equation 2}$$

where:

LADD = lifetime average daily dose
C = concentration of PCBs in Clapper Rail flesh (µg/kilogram dry weight)
IR = intake rate (gram/day)
ED = Exposure duration (years)
BW = body weight (kilogram)
LT = lifetime (years)
Slope = USEPA derived slope factor appropriate for food chain exposure.

Calculations were based on a hunting season from September through December with a 15 bird/day bag limit. This essentially provides 24 hunting opportunities during the season, since birds are usually hunted over a 3-d period during the full-moon high tide (two tides per 24 hr period).

Analysis of Polychlorinated Biphenyls as Aroclor 1268

PCBs were extracted from the tissue using ultrasonic extraction (EPA Method 3550B). Muscle tissue was used from adults but the whole hatchling was ground since they were too small to dissect individual tissues. Tissues were freeze dried and macerated prior to extraction. Dibromooctofluorobyphenyl and tetrachlorometa zylene added as internal surrogate standards. The extractions were performed by sonicating the tissues in 150 ml of acetone: hexane (1:1v/v) using a Tekmar sonic disruptor operated at 100% power in the pulsed mode with a 50% duty cycle for 3 min. The mixture was filtered and the extraction repeated twice with fresh solvent. The combined solvent extracts were dried with Na_2SO_4 solvent, exchanged, and concentrated. Lipids were removed by treatment with 1:1 sulfuric acid solution and the solution back-extracted into hexane. The aqueous phase was discarded and the procedure repeated until a clear hexane extract was obtained. The hexane extracts were concentrated to about 1 ml and then charged onto a pre-cleaned silica gel column to isolate the PCBs from other organic contaminants. The column was sequentially eluted with a series of organic solvents and the PCB fraction collected. The isolated fraction was then concentrated and analyzed using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

PCB analyses were performed on a gas chromatograph equipped with an electron capture detector (ECD), splitless injection, electronic pressure control (EPC), and autoinjector. Separation of PCB congeners was achieved using a 30 m DB-5 (0.025 mm I.D., 0.25 mm film thickness) capillary chromatographic column (J & W Scientific, Folsom, CA). Samples were quantified as Aroclor 1268 using a five-point calibration curve derived from dilutions of certified standards. Six characteristic peaks were selected from the Aroclor mixtures. All selected congener

peaks were at least 25% of the highest Aroclor component. A Hewlett Packard 5890 Series II gas chromatograph with splitless injection, EPC, and a 5972 mass spectrometer (GC-MS) was used to confirm GC-ECD identifications. All samples were analyzed by GC-MS using the selected ion monitoring (SIM) acquisition mode. Selected samples were also analyzed using full scan acquisition in a separate sample injection/analysis. All of the 12 congeners in the Aroclor 1268 mixture were determined in the GC-MS analysis. Selected ions in the SIM mode for different retention time windows were determined from the analysis of an Aroclor 1268 standard. Analysis of spectra obtained in the full-scan mode (mass 50–550) were performed by comparing the mass spectra with Aroclor 1268 standards as well as the National Institute of Standards and Technology (NIST) reference library.

Metal Analyses

Wet tissue and eggshell samples were digested with nitric acid and hydrogen peroxide using microwave digestion protocols. Approximately 25 mg of homogenized sample was placed in a Teflon microwave digestion vessel to which 5 ml of redistilled 70% HNO_3 was added. The vessel was capped and microwave digested using a variable powered program with increasing microwave power applied over 1 hr. After cooling, the vessels were uncapped and 1 ml of 30% H_2O_2 was added; the vessels were then recapped and subject to an identical microwave heating procedure. After the vessels had cooled the digest was brought to a final volume of 25 ml using volumetric flasks. Two duplicate samples, one blank and two standard reference materials (SRMs; DORM-2 and DOLT-2 NRC-CNRC, Ottawa, Ontario, Canada) were included per digestion set. Analysis data with <95% SRM recovery was the rejection criteria. No samples fell below this range. The digested tissue samples were analyzed for V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Sb, Pb, and Hg following the methodology outlined in EPA method 6020. Quality-control procedures were based on EPA SW-846.

Statistical Analyses

A chi-square test was used to determine if DNA strand breakage differed between the contaminated LCP site and the reference locations. Logistic regression (Hosmer and Lemeshow 2000) was used to determine which toxicants (Aroclor 1268 and metals) contributed to DNA strand breakage. All metal concentrations were log-transformed prior to analysis to meet assumptions of normality. A response variable of one was used for observations that had strand breakage and a response variable of zero was used for observations that had no strand breakage. A full model was fit initially and independent variables were removed one at a time based upon their beta values. At each step the corrected Akaike information criteria (AIC) was calculated and compared to the previous model. The model fitting was stopped when AIC was not smaller than the previous model. A randomization function was employed as the statistical validation procedure to evaluate the final logistic regression model's prediction strength (Manly 1998). The leave-one-out cross-validation procedure was used to produce the predicted binomial observation (0 vs. 1) by dropping the data of one observation from the dependant variable and reestimating the response from the tested model (Neter et al. 1990). The observation was then put back into the data set and the procedure was repeated until all observations were used. The model's validity was then judged by comparing the number of accurate predictions to the number of inaccurate predictions.

The relationship between eggshell thickness and FWHM as measures of eggshell structural integrity was explored using a simple correlation as well as a principal component analysis (PCA). Further, the relationship between the toxicants found in the eggshell was also quantified using a PCA. All metal concentrations were log-transformed prior to analysis to meet assumptions of normality. Each measure of integrity was independently tested to determine if they differed based on site using a t-test. A general linear model was then employed to determine if eggshell integrity was dependent upon toxicant load using the principal components from the respective PCA as the response and dependent variables. The first principal component for eggshell integrity was used as the response variable. Site (LCP vs. Blythe Island) was used as an additional dependent variable along with appropriate interaction terms within the model. All analyses were performed based on the results of the clutch rather than the individual egg. This was because individuals within a clutch could not be separated during incubation, which made it impossible to determine from which shell a hatchling hatched. Eggshells were analyzed for metals by grinding material from each egg of the clutch into one matrix, and the hatchling was used to measure PCB load since PCBs, due to their lipophillic nature, will accumulate in lipid biomass and not in the predominately inorganic egg shell matrix (Rassussen et al., 1990, Schwartzenbach

et al., 2003). The FWHM and eggshell thickness were averaged to give one observation for each clutch. A full model was fit initially and independent variables were removed one at a time based upon their beta values. At each step the corrected AIC was calculated and compared to the previous model. The model fitting was stopped when AIC was not smaller than the previous model.

RESULTS

The initial replication (four replicates/individual) and multiple imaging, proved unnecessary for site-level comparisons. The distinction between broken and unbroken sample morphology is distinct and replicable (Fig. 1). In every case, if an individual had broken DNA, all four replicates exhibited a broken morphology. Likewise, for individuals with unbroken DNA, all four replicates exhibited no broken morphology.

All 10 birds from LCP (100%) exhibited broken DNA, one of eight birds (12.5%) from Mackay River had broken DNA and one of 12 birds (8.3%) from Troupe Creek exhibited broken DNA. When the birds from Mackay River and Troupe Creek were combined into a single reference sample, two of 20 birds (10%) exhibited broken DNA. Using this frequency to generate the expected values for LCP, a chi-square analysis resulted in a highly significant value ($G_1 = 46.05$, $P = 1.15 \times 10^{-11}$). Thus, at the population level, Clapper Rails from LCP exhibited a significantly higher frequency of double stranded DNA breaks compared to birds from Mackay River and Troupe Creek. The final logistic regression model showed that Hg had a model probability of 99% with a positive relationship between breakage and Hg concentration while the probability for Pb was 1% and had a negative relationship between breakage and Pb concentration (Table 1). No other metals showed significant relationships (Body burdens for all metals in adult clapper rails are listed in Appendix 1 and for PCB levels in adults and hatchlings in Appendix 2). The take one out cross validation procedure showed that the

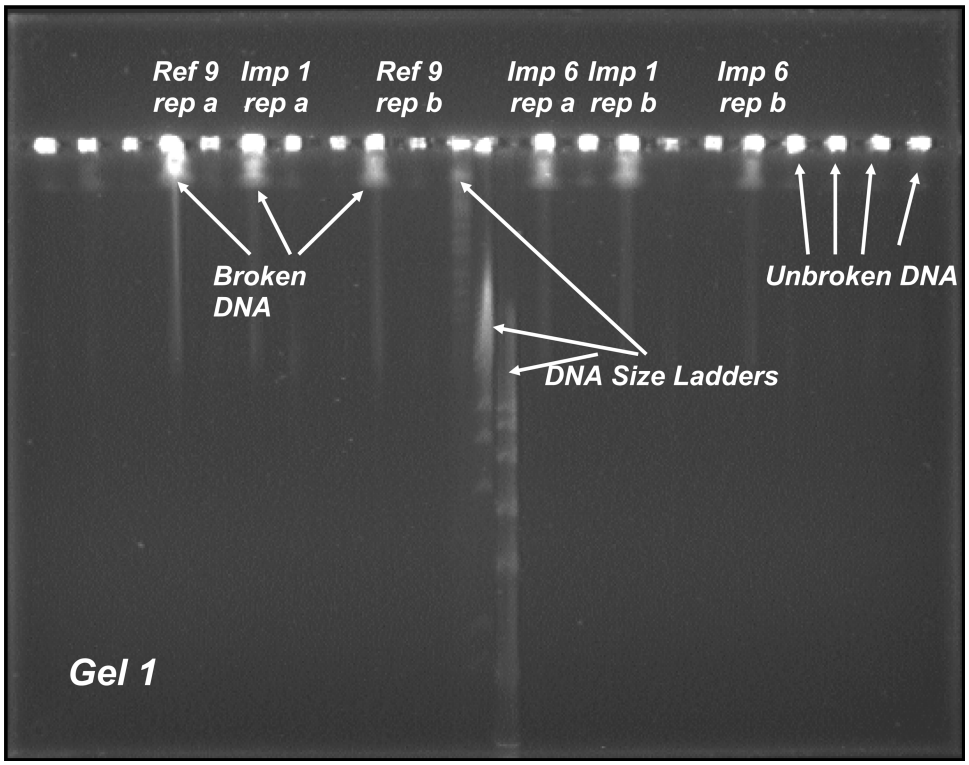

FIGURE 1. Example of broken, unbroken, and DNA size ladders used on a gel run to measure DNA strand breakage in adult Clapper Rails. This image shows that the distinction between broken and unbroken sample morphology is distinct and replicable. Ref refers to the reference site and Imp the impacted site. The numbers refer to individual birds collected from each site and rep a and rep b refer to replicate samples run on the same gel. Thus Imp 1, rep a refers to the first sample from the first bird collected from the impacted site.

TABLE 1. RESULTS OF THE LOGISTIC REGRESSION ANALYSIS OF DNA STRAND BREAKAGE AND CONTAMINANT CONCENTRATION.

Parameter	df	Estimate	SE	χ^2	P	POC (%)
Intercept	1	0.490	3.351	0.021	0.8836	
Hg	1	20.469	9.795	4.367	0.0366	99.086
Pb	1	-4.255	3.271	1.692	0.1933	0.914

Note: POC is the probability of change computed from the odds-ratio.

logistic model predicted 10 of the 12 broken samples correctly and 17 of the 18 unbroken samples correctly.

Thirty-four nests (21 from reference areas and 13 from LCP) comprising 146 eggs were used for eggshell integrity-toxicant analyses. Thickness and FWHM display a negative correlation ($r = -0.733$, $P < 0.0001$, $N = 34$). Further, there was no difference between the LCP and the reference locations for either measure. The PCA showed that the first component explained 87% of the variation for the two measures. The PCA for metals in the eggshell and PCB's in hatchlings (Appendix 2) indicated that 79.1% of the total variation was contained in the first four components (Table 2). The simple linear regression showed that PC2 (mostly explained by Mn, Zn, Hg, and PCB) was a significant variable as was the interaction of site and PC4 (mostly explained by Cu and Zn) (Table 4; Fig. 3). Site, PC1 and PC4 were left in the final model because of constraints imposed when using PC components and from significant interaction terms.

Additional lifetime human cancer risk from consuming PCB contaminated birds over a 30-yr period for a 70 kg adult using the maximum PCB value from birds collected from LCP and reference areas were both above the 1×10^{-6} risk (expectation for unexposed individuals) threshold used by the EPA to determine risk (Table 5). Specifically, the additional lifetime cancer risk for LCP was 1.41×10^{-3}, while the general Brunswick area was lower with the estimated risk being 1.37×10^{-4}.

TABLE 2. PRINCIPAL COMPONENT ANALYSIS (PCA) OF THE METALS MEASURED IN THE EGGSHELLS FROM EGGS COLLECTED AT THE LPC AND REFERENCE SITES—EIGENVALUES FOR THE 11 PCS CALCULATED FOR THE METALS.

PC	Eigenvalue	POV	CPOV
1	**3.36697651**	**0.3061**	**0.3061**
2	**2.68185851**	**0.2438**	**0.5499**
3	**1.39326853**	**0.1267**	**0.6766**
4	**1.25509985**	**0.1141**	**0.7907**
5	0.72052911	0.0655	0.8562
6	0.67220944	0.0611	0.9173
7	0.34738532	0.0316	0.9488
8	0.21722518	0.0197	0.9686
9	0.15444438	0.0140	0.9826
10	0.12547491	0.0114	0.9940
11	0.06552826	0.0060	1.0000

Notes: The PCA was based upon the correlation matrix. Significant PCs are in bold. POV is the proportion of variance explained by each component, and CPOV is the cumulative proportion of variance explained by the component and all previous components.

DISCUSSION

Aroclor 1268, as well as other toxicants, are bioavailable in the Brunswick estuary. The DNA strand breakage study and the PCB additional lifetime cancer risk estimates showed that these anthropogenic insults are impacting the estuary at both the landscape and local scale. During the late fall and early winter, Clapper Rails have larger home ranges than during their breeding season (Meanley 1985). Further, although it is thought that the Brunswick population is non-migratory, this winter population could also have been mixed with other migratory populations from the north. Regardless, the group-level DNA strand breakage analyses showed that birds that were collected and assumed to reside in and around the LCP site had a higher percentage of individuals with broken DNA compared to those collected from other areas only a few kilometers away. One possibility is that birds collected from the LCP site were resident birds that may have spent large amounts of time in the areas, possibly even breeding at that site. Another possibility is that the birds may have only used that area for over wintering, which would imply that the contamination in that marsh might accumulate and show toxicity response very quickly.

The results from the logistic regression analysis indicate that Hg is primarily responsible for increased levels of strand breakage. However, it is also possible that breakage can be elevated due to the synergistic effects of a contaminant mixture that would not be readily detectable without much larger sample sizes. One of the birds collected from the reference sites that was scored as having broken DNA, had levels of Hg in the same range as birds from LCP and the other did not. This individual-level analysis helps explain one data point from our group-level analysis but also gives pause in ascribing too much weight to our result of Hg and breakage. While we can certainly state that Hg concentrations are influencing the levels of strand breakage we have less confidence in stating that

TABLE 3. PRINCIPAL COMPONENT ANALYSIS (PCA) OF THE METALS MEASURED IN THE EGGSHELLS FROM EGGS COLLECTED AT THE LCP AND REFERENCE SITES—COMPONENT LOADINGS FOR THE FOUR SIGNIFICANT EIGENVECTORS.

Variable	PC1	PC2	PC3	PC4
Ni	0.157079	0.257248	**0.363281**	0.264882
Mg	-0.134219	0.314552	**0.544807**	0.257439
Al	**0.484737**	0.065041	0.203060	-0.113849
P	**-0.391599**	0.239709	0.218198	-0.252857
Mn	**0.352640**	**0.354443**	-0.052157	-0.249082
Fe	**0.483298**	0.117830	0.169759	-0.184244
Cu	-0.103615	0.217930	-0.045241	**0.676402**
Zn	-0.215307	**0.417834**	0.201922	**-0.342930**
Pb	**0.345458**	-0.128209	0.086392	0.322304
Hg	-0.069768	**0.470641**	**-0.423272**	0.062197
PCB	0.172501	**0.416722**	**-0.470519**	0.088362

Note: Loadings with an absolute value >0.33 are in bold.

TABLE 4. GENERAL LINEAR MODEL ANALYSIS TO DETERMINE EGGSHELL INTEGRITY DEPENDENCY UPON MEASURED PARAMETERS.

Effect	df	F	*P*
Site	1	0.70	0.411
PC1	1	3.74	0.065
PC2	1	9.87	0.004
PC4	1	0.84	0.369
Site × PC4	1	7.69	0.011

Notes: The response variable of the regression is the principal component of eggshell integrity variance. The dependent variables are site (contaminated vs. reference) and the principal components of the PCB levels from the hatchlings and the metal levels within the eggshells.

other contaminants in the mixture have little or no effect.

It is also unclear what affect increased levels of double-strand breakage may have on the rails on either an ecological or evolutionary time scale. This will be determined by the extent of the breakage, the specific tissue it occurs in and the efficiency and quality of the repair. In somatic cells, if the break is not repaired it will most likely lead to a loss of cell function and depending on the tissue, apoptosis (Rich et al. 2000). If it is repaired with error then the mutated cell may become cancerous (Kasprzak et al. 1999). It is unlikely, that levels of cell death could be high enough or rates of cancer production fast enough to influence survival rate for Clapper Rails in the wild. However, the increased energy demands of these processes may have measurable effects on both survival and reproduction under stressful conditions (Hoffmann and Parsons 1991). In gametic cells, repair mechanisms that result in mutations will increase the base mutation rate of progeny and thus has the potential to change the evolutionary trajectory of populations and species (Fox 1995). Recent research has implicated multiple mechanisms in vertebrates for the repair of double-strand breaks (Liang et al. 1998), some also being involved in translocation events (Kanaar et al. 1998) and generation of antibody variability (Karran 2000). Thus, the evolutionary effects of double-strand breaks may be more pervasive than expected.

All birds that were collected did have measurable PCB levels (Appendix 2), which can be judged by the calculations to estimate the additional lifetime cancer risk from consuming PCB contaminated flesh (Table 5). Measurable levels of Aroclor 1268 as well as metals have been found in the soil and rail food items in both the LCP and reference areas (J. M. Novak et al., unpubl. data), indicating that this toxicant is bioavailable at the landscape level. Therefore, it is likely that birds collected from these reference areas are picking up Aroclor 1268 from the respective areas. However, because these birds are likely to have winter home ranges that may encompass the reference areas as well as LCP, they may have accumulated the PCB from the LCP site itself. Home-range studies of resident rails are needed to address these questions.

TABLE 5. ADDITIONAL LIFETIME HUMAN CANCER RISK FROM CONSUMING PCB-CONTAMINATED CLAPPER RAILS.

Site	PCB concentration (μg/kg)	Ingestion rate (g/d)	Lifetime average daily dose (mg/kg -d)	Risk
LCP	1.76×10^{-02}	6.58	7.07×10^{-04}	1.41×10^{-03}
Reference	1.70×10^{-03}	6.58	6.85×10^{-05}	1.37×10^{-04}

Notes: From areas near the LCP site (N = 10) and reference locations (N = 17) in the Brunswick area. Calculations were based on a 30-yr exposure over a 70-yr lifetime for a 70-kg adult using a USEPA derived slope factor of 2 for the maximum PCB levels found from each site.

Since eggshell integrity should be indicative of the toxicity of contaminants at both the local and landscape level it can be a useful ecological endpoint. Clapper Rails in the southeastern US will start to set up breeding territories in early February and their home ranges will focus around those areas through the breeding season (Meanley 1985; J. M. Novak et al., unpubl. data). Therefore, the toxicants those female birds accumulate in the months before breeding should be representative of the area where they breed. However, toxicants that have been accumulated prior to the nesting season from other areas may still be persistent in the birds' organs and therefore depurated into the egg as well. The integrity of some of eggshells from the LCP site as well as the reference areas did show signs of structural problems. Although the nature of the matrix of contaminants is extremely complex, some interesting interpretations of the data can be made. For example, since the plot of the principal component 2 from the PCA showed that individuals with higher Mn, Zn, Hg, and Aroclor 1268 (PCB) had thinner eggshells that were less oriented and that the trends were the same from both the LCP and reference sites, may imply that ecosystem integrity has been compromised for the entire Brunswick estuary (Fig. 2). That is, the bioavailability of Aroclor 1268 at the Brunswick landscape level coupled with the possibility of birds from the reference areas using the contaminated site during the winter, contributed to the structural problems found within the eggshells. In summary, since the slopes of the lines did not differ between the sites, the effect of these contaminants appears to be at a scale greater than the individual sites themselves.

Another interesting finding is that principal component 4 has a significant interaction with site (Fig. 3). In this case, higher levels of Cu and lower levels of Zn are associated with stronger eggs at the reference site but weaker eggs at LCP. This most likely indicates localized effects at each site that mitigates the relationship between these toxicants and eggshell integrity. Since the contaminants represent a very complex mixture, it is unlikely these relationships can be further disentangled without using an experimental approach. It is difficult to speculate how these findings would have influenced egg survival in the wild. Although more oriented eggshells have less integrity, the greater thickness may compensate for this flaw. This portion of the study was inspired by the fact that when eggs were marked with a pencil, some tended to quickly shatter even with the lightest touch of a pencil, indicating structural problems with the eggs. Further study of the possible compensatory nature of eggshell

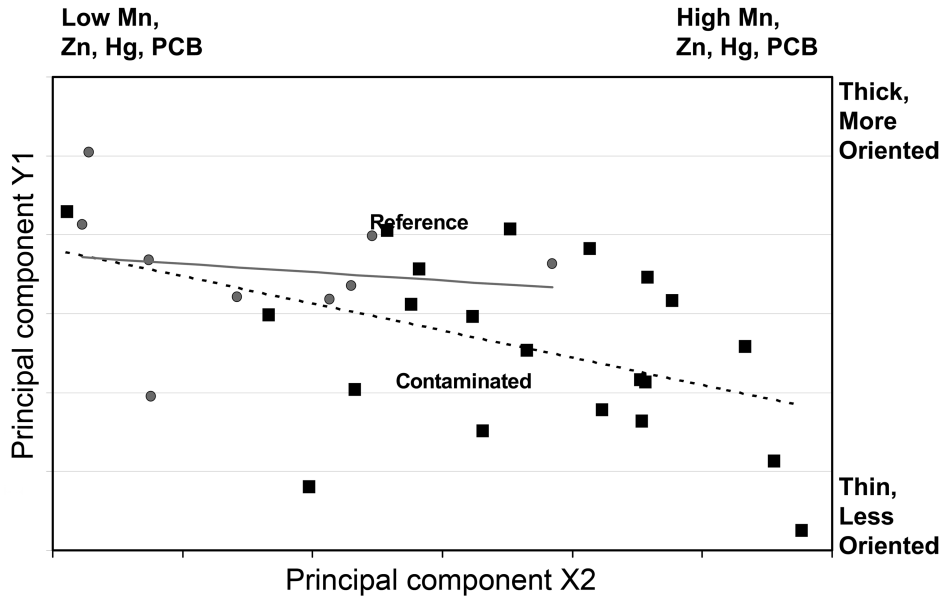

FIGURE 2. Plot of principal component Y1 (from the eggshell thickness and orientation PCA) vs. X2 (from eggshell and hatchling contaminant level PCA, Table 3) showing that for both the reference (circles) and impacted (squares) location, individuals with higher Mn, Zn, Hg, and Aroclor 1268 (PCB) had thinner eggshells that were less oriented.

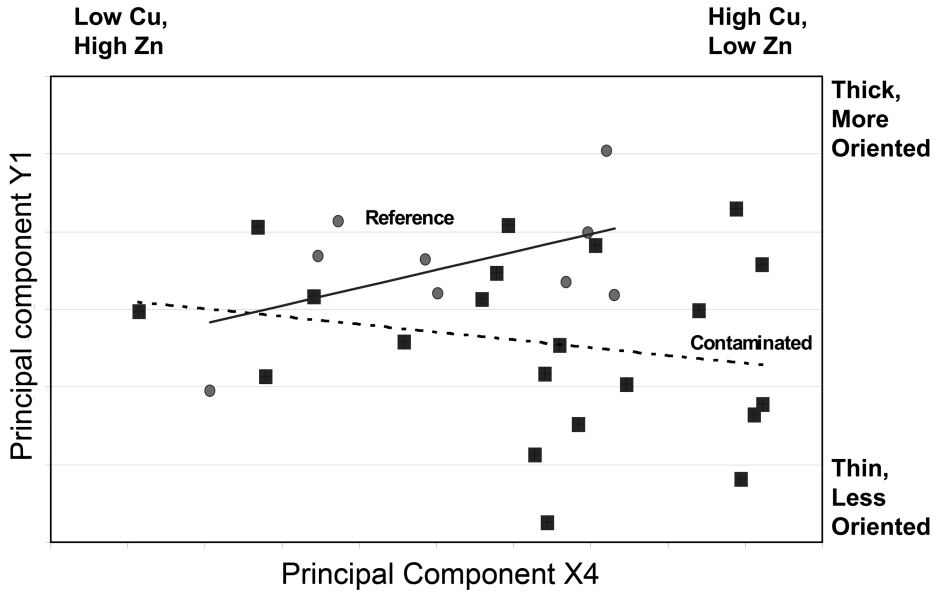

FIGURE 3. Plot of principal component Y1 (from the eggshell thickness and orientation PCA) vs. X4 (from eggshell and nestling contaminant level PCA, Table 3) showing that higher levels of Cu and lower levels of Zn are associated with stronger eggs at the reference site but weaker eggs at the impacted site.

integrity with crystal orientation must be pursued to strengthen the use of these measures as endpoints of survivability.

Estimating the additional lifetime cancer risk from consuming PCB contaminated meat is the most widely used and easy to understand measure for a human toxicological endpoint. Further, cancer studies comparing commercial and environmental mixtures, especially those found in the food chain, warn that food chain risks could be underestimated (Environmental Protection Agency 1992). In addition, PCBs have been shown to cause a variety of health effects to humans and other animals beyond carcinogenicity. Specifically, studies have revealed that PCBs can cause a variety of immune, reproductive, neurological, as well as endocrine effects (Environmental Protection Agency 1992). It is unclear how environmental processes alter the composition and subsequent toxicity of PCB mixtures and exposure to a myriad of contaminants can further complicate interpretations of PCB toxicity as shown in this study. Our data do show that Aroclor 1268 is bioavailable throughout the Brunswick estuary, and poses a potential health risk to individuals who may consistently ingest Clapper Rail flesh.

Since Clapper Rails are an integral part of the ecosystem structure of the Brunswick estuary, their use as indicator species can be very helpful in understanding how anthropogenic activities can affect estuarine systems. Based on two separate ecological endpoints, it appears that the contamination by metals and PCBs in the estuary is having detrimental effects on the resident Clapper Rail population. Further, based on the PCB levels alone, it appears that those who consistently hunt Clapper Rails in this region should consider hunting outside the immediate Brunswick area to avoid possible detrimental health effects. The information and techniques outlined in this paper can be used as a template for other regions of the US that have large rail populations, to use these species as ecological indicators. DNA strand breakage and eggshell integrity offer an additional value in that they are non-lethal techniques and therefore can be used to study threatened and endangered populations.

ACKNOWLEDGMENTS

We thank K. Hastie and G. Masson for logistical help. We would also like to thank S. Boring, P. Shull, and S. Murray for help in the field. Two anonymous reviewers provided insight that improved the quality of this manuscript. This project was funded through the USDI Fish and Wildlife Service and Financial Assistance Award DE-FC09-96SR18546 from the U.S. Department of Energy to the University of Georgia Research Foundation.

APPENDIX 1. CONTAMINANT BURDENS (PPM WET WEIGHT) FOUND IN ADULT CLAPPER RAIL MUSCLE.

LCP (N=10)	Al	Cr	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Rb	Sr	Cd	Ba	Pb	Hg
Mean	8.17	0.36	0.46	62.53	0.02	0.19	3.16	12.42	0.28	0.32	2.03	0.32	0.01	0.09	0.11	1.40
Standard error	2.05	0.03	0.05	6.06	0.01	0.04	0.31	1.67	0.03	0.06	0.05	0.06	0.00	0.03	0.01	0.21
Median	7.00	0.34	0.43	54.97	0.01	0.15	3.13	10.89	0.29	0.38	2.05	0.27	0.01	0.05	0.12	1.16
Standard deviation	6.48	0.10	0.16	19.16	0.02	0.11	0.97	5.28	0.10	0.18	0.15	0.18	0.01	0.11	0.04	0.67
Minimum	0.00	0.25	0.26	47.32	0.01	0.09	1.80	8.48	0.11	0.00	1.79	0.15	0.00	0.03	0.05	0.61
Maximum	22.43	0.60	0.79	108.69	0.07	0.44	5.62	26.62	0.42	0.50	2.24	0.75	0.02	0.39	0.16	2.52

Reference (N = 20)	Al	Cr	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Rb	Sr	Cd	Ba	Pb	Hg
Mean	6.48	0.44	0.45	71.72	0.06	0.91	3.81	12.05	0.52	0.38	2.23	0.26	0.01	0.05	0.19	0.44
Standard error	0.96	0.03	0.02	3.90	0.02	0.26	0.29	0.62	0.06	0.04	0.08	0.02	0.00	0.01	0.03	0.03
Median	7.32	0.42	0.45	67.62	0.02	0.24	3.44	11.67	0.45	0.38	2.28	0.24	0.01	0.04	0.16	0.41
Standard deviation	4.30	0.13	0.07	17.46	0.08	1.16	1.28	2.79	0.28	0.16	0.36	0.09	0.01	0.03	0.14	0.11
Minimum	0.00	0.26	0.29	44.38	0.01	0.08	2.15	8.77	0.20	0.00	1.68	0.13	0.00	0.02	0.04	0.27
Maximum	12.87	0.73	0.56	105.62	0.31	3.98	6.10	21.33	1.23	0.66	2.87	0.49	0.03	0.12	0.54	0.72

APPENDIX 2. SUMMARY STATISTICS OF THE AMOUNT OF AROCLOR 1268 (PPM DRY WEIGHT) FOUND IN ADULT CLAPPER RAIL MUSCLE COLLECTED FROM THE LCP MARSH AND REFERENCE AREAS IN BRUNSWICK, GEORGIA, DURING THE 1999 HUNTING SEASON AND IN CLAPPER RAIL HATCHLINGS IMMEDIATELY AFTER HATCHING THAT WERE COLLECTED AS EGGS FROM THE LCP MARSH AND THE BLYTHE ISLAND REFERENCE AREA IN BRUNSWICK, GEORGIA, DURING THE 2000 NESTING SEASON. HATCHLING CONCENTRATIONS WERE AVERAGED FOR EACH NEST TO PROVIDE COMPARISONS FOR EGGSHELL ANALYSES.

	Adults		Hatchlings	
Statistic	LCP	Reference	LCP	BLYTHE
Mean	6.7619	0.4308	147.3442	31.8828
Standard error	2.0016	0.1243	19.1795	6.3084
Median	3.9930	0.3000	81.7666	25.7578
Standard deviation	6.0048	0.5124	144.8020	26.7641
Minimum	1.3200	0.0030	13.9373	9.2856
Maximum	17.5600	1.7020	659.6327	114.1756
N	9	17	57	18