Songbirds do not create long-term stores of calcium in their legs prior to laying: results from high-resolution radiography

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SUMMARY

We prepared high-resolution radiographs of the leg bones of three passerine species using X-rays at the Cornell High Energy Synchrotron Source (CHESS), which allows imaging with exceptionally high contrast and resolution. In studies of living tree swallows before and after laying and in studies of brown-headed cowbirds and great tits collected before, during or after laying, there is no evidence of calcium storage in the leg bones. By contrast, great tit nestlings raised in environments with extremely low calcium availability exhibited calcium deprivation in the development of their leg bones.

1. INTRODUCTION

During egg laying, birds must mobilize large amounts of calcium (Ca) for the formation of their eggshells. Observations on chickens and pigeons (Simkiss 1961, 1967; Gilbert 1983) have suggested that the formation of large amounts of 'medullary bone' in the hollow lumens of bones, especially those of the legs, could serve as a store of easily mobilized Ca. To our knowledge, the only detailed studies of medullary bone in passerine birds were conducted in the 1930s on house sparrows (*Passer domesticus*) (Kirschbaum *et al.* 1939; Pfeiffer *et al.* 1940), and they suggested that medullary bone was only present when the 'ovary was near maximal activity' (Kirschbaum *et al.* 1939). All other studies of the possibility of calcium storage in laying passerines are based on comparisons of bone masses of birds collected before and after laying (Ankney & Scott 1980; Graveland 1995; Houston *et al.* 1995; Krentz & Ankney 1995). There are commonly thought to be two basic strategies of resource use in birds: 'capital' breeders accumulate resources over a long period prior to reproduction, and 'income' breeders who do not store resources but lay eggs on resources that are ingested very shortly before egg laying (Drent & Daan 1980; Thomas 1988; Winkler & Allen 1996). Greater understanding of these strategies has become of more than basic interest in recent years, as increasing evidence indicates that reproductive failures in some passerines can be linked to decreased calcium avail-

ability in their laying environments, due in part to the effects of acid rain (Graveland *et al.* 1994; Graveland & van der Wal 1996).

2. MATERIALS AND METHODS

To directly assess the temporal dynamics of leg-bone Ca stores, we compared radiographs of legs from tree swallows (*Tachycineta bicolor*), brown-headed cowbirds (*Molothrus ater*) and great tits (*Parus major*). In the study of *Tachycineta* we conducted a longitudinal study of females nesting at the Cornell Experimental Ponds. (For further details on the study area and ecology of this population see Winkler (1994) and Winkler & Allen (1996).) To control for potential differences between individuals, seven females were captured before dawn roosting in nest-boxes from 9–22 days ($\bar{x}$ = 13.7), before laying and again 17–25 days ($\bar{x}$ = 22.7) after the completion of their clutches. On each of these occasions the birds were brought to CHESS for radiography; they were measured, weighed and returned to their breeding area within 2 h of capture. All females laid eggs and subsequently reared young with the same success as groups of control females that were not exposed to radiation and that were not captured before laying.

Brown-headed cowbirds are brood parasites, and, as such, they are known for extraordinary egg-laying capacities (Ankney & Scott 1980; Jackson & Roby 1992). To further assess the importance of long-term skeletal calcium stores, we compared radiographs of leg bones from female cowbirds collected in the eastern U.S. before laying with those of females collected during or after laying. We also compared female leg bones with those of males, which have no adaptive need for large calcium stores. These leg bones, from the Cornell University Vertebrate Collections, had been prepared with dermestid beetles, and they had not been subject to boiling or chemical treatment.

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Figure 1. Comparison of radiographs from leg bones of *Tachycineta bicolor* (tree swallow) using (A) a conventional X-ray source and (B) monochromatic synchrotron radiation. The conventional image was taken at the Cornell School of Veterinary Medicine with a GX-650 X-ray generator (Picker International, Inc., Cleveland, Ohio). For this exposure the X-ray generator was operated at 55 kV and 300 mA, producing characteristic tungsten fluorescence at 8.4 keV; the low energy part of the broad Bremsstrahlung spectrum was reduced by a 0.5 mm aluminium filter. The image was captured on high resolution Kodak X-Omat TL film. For methods used at CHESS see text. The superior image quality obtained with synchrotron light is apparent.

Finally, we took a series of radiographs of leg bones from great tits that were collected or salvaged from birds in The Netherlands, living in habitats of different calcium availability (Graveland *et al.* 1994; Graveland 1995). We contrasted the bones of females collected in forests of normal Ca availability with 'calcium-deprived females' from the Buunderkamp forest (Graveland 1995). We also compared the bones of fledglings that were raised in captivity by parents that were provided with highly deficient calcium supplies ($< 0.1\%$ of the diet) versus those that were raised in areas of high calcium availability, or in captivity by parents provided with ad lib calcium. These fledglings all died of natural causes at 19 days of age. All tit bones were preserved only by freezing prior to X-ray analysis, thus avoiding any chemical treatments of the bones.

The experimental set-up, optimized for high resolution X-ray imaging, utilized a double-crystal monochromator with asymmetric Si-220 reflection, producing X-rays of 12 keV with high energy resolution ($\Delta E/E$ better than 10^{-5}). Radiographs showing lateral views of the bone samples were

stored on high resolution X-ray film (from a single batch of Kodak Industrex M). Exposure times were controlled by monitoring the incident intensity of photons during sample irradiation. The collinearity and monochromatization of synchrotron radiation eliminate distortions present in images made with conventional X-ray sources (figure 1), providing higher spatial contrast and improved sensitivity for chemical elements. For experiments with live birds, the high intensity of the beam (3×10^9 photons s^{-1}) allows for short exposure times, typically 0.3 s, avoiding image blur due to specimen movement. From a calibrated $CaCl_2$ absorption standard (Witt & Cameron 1970), we determined the sensitivity to bone minerals in our experiments to be better than 0.010 g cm^{-3} .

The film images were digitized at 16-bit pixel depth to a spatial resolution of $9\text{ }\mu\text{m}$, with the limit of resolution determined by the film scanner. One of us (R.P.) conducted all measurements of bone density, and they were done blind to the origin of each sample, with reference only to the specimen number. Digital images were analysed using NIH

Image (Rasband 1996), and bone densities were compared with *t*-tests using SYSTAT (SYSTAT 1992). All estimates of bone density were based on circular areas of equal size within samples for each bone area in a given species. In all samples, the size of this area was chosen so as to avoid edge-on views of the bone walls.

3. RESULTS AND DISCUSSION

There were no detectable differences in the thickness, structure or density of the leg bones of female tree swallows taken before and after egg laying (figure 2; table 1). There is therefore no evidence in our samples that females built up large stores of calcium in their leg bones in preparation for the calcium demands of laying. Indeed, the results indicate that a larger sample of females may actually have revealed higher Ca levels in the post-laying period. Such a result might be explained by increased Ca sequestration immediately before and during laying, but any such increase in Ca less than 9 d in advance of laying could not be construed as 'long-term' storage (see Winkler & Allen 1996). A whole-body radiograph of a female tree swallow collected during egg laying (figure 2) shows that the largest single depot of calcium in the body during egg laying is found in recently ingested food in the gizzard, not in any of the bones.

Analysis of the densities of leg bones of female cowbirds collected before and during laying (table 1) leads to the same conclusion as was drawn from the swallow studies: these bones show no sign of thickening or of development of medullary bone in preparation for the breeding season (cf. Ankney & Scott 1980), nor are female leg bones distinguishable from those of males. As in the swallow results, there is a weak indication that bone densities in laying cowbirds may actually be higher than those in pre-laying females. Such a finding would contrast with an earlier study of changes in bone

mass in this species (Ankney & Scott 1980). Further detailed studies, including X-ray investigations of leg bone changes with improved statistical power, are necessary to resolve the Ca dynamics of cowbirds.

The third sample, leg bones from great tits collected or salvaged from Ca-rich and Ca-poor habitats, showed more variations among individual birds than we detected in the other two studies; however, there were no significant overall differences between the groups of adults breeding in Ca-poor and Ca-rich environments, respectively (figure 3*a* versus figure 3*b*; table 2). In contrast, there were substantial differences in the leg bones of great tit young: chicks reared under conditions of high Ca availability (figure 3*c*) had substantially thicker bones than did those reared under Ca-deprived conditions (figure 3*d*). Many of the juveniles raised in Ca-poor environments suffered broken and deformed legs which would have crippled these birds permanently even if they had lived longer. These results indicate that when environmental Ca supplies are low, great tit adults allow reproductive functions to deteriorate rather than deplete the somatic Ca depot in their skeletons.

This study has demonstrated the feasibility of using synchrotron radiation for calcium measurements of unprecedented spatial resolution in collected, as well as living birds. Radiography has allowed us to determine with a fair degree of certainty that passerines do not store large amounts of calcium for eggshell production in their leg bones more than a few days in advance of laying. Further studies involving complementary techniques are needed to resolve the extent to which medullary bone may be involved in the short-term calcium dynamics of laying cowbirds, but even if it is involved as a short-term store it is not likely to be a large storage depot (Ankney & Scott 1980; Graveland 1995). Rather, it appears increasingly clear that passerines must base all or most of the production of

Figure 2. 'Whole-body' radiograph from *Tachycineta bicolor* (tree swallow) taken with a conventional X-ray source using the same method as described in the caption for figure 1. The insets show typical synchrotron radiographs from *Tachycineta bicolor* before (left) and after (right) egg laying.

Table 1. *Comparisons of bone densities from live tree swallows before and after laying, and from collected brown-headed cowbirds before and during, or after laying*

(For swallows, the estimates for bone density are the mean pixel values (after background subtraction) from analyses of at least 3000 pixels within each region of interest. Note that although the tests for the two tibiotarsal scores approach significance, the differences are opposite in sign to what would be predicted by long-term storage of calcium for egg laying in the leg bones. For cowbirds, females collected on or before 1 May were assumed to be 'pre-laying'. Females collected later were assumed to have been collected during laying. The estimates of bone densities are the mean pixel values (after background subtraction) from analyses of at least 12000 pixels at each site of the bones. The *p*-values presented for all comparisons are based on separate variances *t*-tests run with the SYSTAT module in SYSTAT (SYSTAT 1992).)

Species/site of measurement	mean	s.D., <i>n</i>	mean	s.D., <i>n</i>	<i>p</i>
females					
tree swallow					
distal tibiotarsus (cranial)	55.62	9.17, 7	66.89	14.35, 7	0.07
distal tibiotarsus (caudal)	33.16	7.81, 7	41.31	14.32, 7	0.07
mid-tibiotarsus	80.91	32.14, 7	78.57	13.46, 7	0.84
females					
brown-headed cowbird					
mid-femur	124.44	17.65, 3	143.33	14.06, 3	0.22
distal femur	132.11	40.26, 3	170.95	24.45, 3	0.24
proximal tibiotarsus	130.13	7.17, 3	156.81	39.92, 3	0.37
mid-tibiotarsus	178.11	17.98, 3	177.77	20.13, 3	0.98
females					
mid-femur	133.89	17.63, 6	117.46	29.37, 4	0.37
distal femur	151.53	36.61, 6	139.87	9.63, 4	0.48
proximal tibiotarsus	143.47	29.52, 6	142.12	8.02, 4	0.92
mid-tibiotarsus	177.94	17.07, 6	186.81	26.31, 4	0.58
males

Figure 3. Radiographs of the distal ends of femurs from *Parus major* (great tit): (A) and (B) adult females collected from calcium-rich and calcium-poor habitats, respectively; (C) and (D) juveniles of the same age raised in captivity simulating calcium-rich and calcium-poor habitats, respectively.

Table 2. *Estimates and comparisons of bone density taken from three sites in the leg bones of female great tits collected during the breeding season in the Netherlands*

(The estimates for bone density are the mean pixel value (after background subtraction) from analyses of at least 12000 pixels at each site of the bones. Passerine femurs have three facets on their distal ends, the central one of which projects farther distally. 'Distal femur' measurement was taken from within the distal-most facet of the femur, and the 'near distal femur' measurement was taken in an area of similar size that sampled a cross-section through all three facets. The *p*-value presented for each comparison is based on separate variances t-tests run with the STATS module in SYSTAT (SYSTAT 1992).)

site of measurement	mean	s.D., <i>n</i>	mean	s.D., <i>n</i>	<i>p</i>
adults					
	Ca-deprived		not Ca-deprived		
mid-femur	169.05	15.17, 7	180.45	34.95, 3	0.63
distal femur	155.94	35.20, 7	164.21	22.16, 3	0.67
near distal femur	156.22	12.39, 7	165.47	40.59, 3	0.73
	problems laying eggs		no problems laying eggs		
mid-femur	165.40	12.63, 4	177.19	25.63, 6	0.37
distal femur	154.97	47.17, 4	160.73	19.00, 6	0.83
near distal femur	157.26	10.98, 4	160.15	28.33, 6	0.83
Juveniles					
	Ca-deprived		not Ca-deprived		
mid-femur	25.16	5.34, 2	93.78	5.34, 2	0.006
distal femur	82.79	20.27, 2	111.85	3.87, 2	0.28
near distal femur	53.07	14.28, 2	80.86	17.21, 2	0.23

egg shells on their income of calcium over short time periods before and during the laying of a clutch (Ankney & Scott 1980; Graveland 1995; Houston *et al.* 1995; Krementz & Ankney 1995). Thus, any environmental changes, such as acid rain, which decrease the availability of this crucial mineral, are likely to have direct and immediate impacts on the reproduction of these birds. Available evidence suggests that there is considerable diversity in the environmental sources of calcium used by passerine birds (Graveland 1995), and better understanding of this aspect of avian biology may have important implications both for our understanding of passerine conservation biology and the evolution of passerine life histories.

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