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UPTAKE OF INGESTED CALCIUM DURING EGG PRODUCTION IN THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*)

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ABSTRACT.—Small passerines forage for calciferous material on a daily basis during egg laying, but beyond this general observation, mechanisms of calcium uptake are poorly understood. I investigated calcium uptake during egg laying in Zebra Finches (*Taeniopygia guttata*) by administering a 5-μCi dose of radioisotopic calcium (^{45}Ca) by proventricular intubation exactly 1 h after oviposition. A nonlaying (control) female was dosed at the same time as each egg layer. Egg layers excreted less of the dose (<0.7 μCi) than controls (>1 μCi) over the entire ovulatory cycle. Egg layers incorporated more calcium into their skeletons than controls during the first 16 h post-dosing, but localization was similar to that of controls 16 to 24 h after the dosing period. Calcium was more than 60 times more abundant in the reproductive tissues of egg layers than in controls 8 to 16 h after the dosing period, suggesting that the majority of egg calcification occurred during this period. The decline in skeletal incorporation of ^{45}Ca 16 to 24 h after dosing may indicate mobilization of medullary-bone reserves to supply the calcium needed to complete shell secretion. Evidence from a number of avian species suggests that daily ingested calcium is essential for egg formation; my results show in quantitative terms the fate of ingested calcium during egg formation in the Zebra Finch. *Received 30 October 1996, accepted 17 March 1997.*

IN MANY SPECIES OF BIRDS, the production of eggs requires a great nutritional investment on the part of the female. For instance, clutch mass in the Blue Tit (*Parus caeruleus*) represents 130% of the female's body mass (Perrins and Birkhead 1983). The production of the shell is a fundamental process of egg formation. The fully formed shell provides protection and a source of calcium for the developing chick (Taylor 1970). The eggshell consists principally of calcite, a crystalline form of calcium carbonate that constitutes 98% of the dry mass of the shell (Romanoff and Romanoff 1949). Little attention has focussed on the source of calcium (or, for that matter, on any of the nutrients; see Perrins 1996) required for egg production in any birds other than domestic poultry. Recent research by Graveland et al. (1994), however, has highlighted the importance of calcium in avian reproduction and how its limited supply can result in marked declines in egg production. Acid deposition leaches calcium from the soil. Particularly in naturally acidified areas, the resultant calcium deficiency has led to the production of thinner eggshells and sometimes to the abandonment of egg laying in some European passerines (e.g. Ormerod et al. 1988, Drent and Woldendorp 1989, Graveland 1990, Carlsson et al. 1991).

In laying domestic hens (*Gallus domesticus*), 125 mg of calcium are deposited every hour. Taylor (1970) calculated that this mobilization represents a total clearance of blood calcium every 12 min. Each eggshell of a domestic hen requires approximately 2 g of calcium. A digestive bottleneck restricts to approximately 1 g per day the amount of calcium that is available from dietary sources (Simkiss 1961), but the shortfall is met by mobilization of calcium from the medullary bone (Common 1933). In extreme cases, as much as 10% of the skeletal mass can be mobilized in less than 24 h. Although medullary bone has been reported in other species (e.g. Northern Bobwhite [*Colinus virginianus*], Ringeon 1940; House Sparrow [*Passer domesticus*], Schifferli 1979, Krementz and Ankney 1995; Mallard [*Anas platyrhynchos*], Landauer et al. 1941; Rock Dove [*Columba livia*], Kyes and Potter 1934), its role as a calcium source during egg production in small birds is poorly understood.

Observations on passerines indicate that

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many (if not all) species collect calcium-rich foods on a daily basis for eggshell production. Specific calcium-foraging behavior has been reported in arctic sandpipers (*Calidris* spp.; MacLean 1974), Blue Tits (Turner 1966); Boreal Chickadees (*Parus hudsonicus*; Ficken 1989), Brown-headed Cowbirds (*Molothrus ater*; Ankney and Scott 1980), Great Tits (*Parus major*; Graveland et al. 1994, Graveland 1995), House Sparrows (Schifferli 1977), Queleas (*Quelea quelea*; Jones 1976), Red-cockaded Woodpeckers (*Picoides borealis*; Repasky et al. 1991), Red Crossbills (*Loxia curvirostra*; Payne 1972, Sušić 1981), and Zebra Finches (*Taeniopygia guttata*; Zann and Straw 1984). The timing of such foraging coincides so precisely with egg laying in these species that it is likely that ingestion of calciferous material is providing much of the calcium required for egg production.

In the Zebra Finch, a five-egg clutch has a total calcium requirement of 89 mg (Donnan 1993, Houston et al. 1995a). Because the total calcium reserve of a female is 90 mg (calculated from mean ash mass), skeletal provisioning of calcium for egg production would necessitate an increase in skeletal reserves of approximately 99% (Donnan 1993). Therefore, endogenous reserves are likely to provide only a minor fraction of the calcium required for egg production in Zebra Finches. In this study, I investigated the incorporation into the tissues and the excretion of ingested calcium in both reproductive and nonreproductive females. The metabolism of ingested calcium was examined at different stages of egg formation to identify how important dietary calcium might be in meeting the calcium requirements for clutch formation in a small passerine.

Methods

Pairs of Zebra Finches were kept indoors in cages measuring 46 × 38 × 30 cm, with two perches on split levels and a single external nesting box. The aviary room was maintained at a constant temperature of 23°C and under conditions of artificial light with a photoperiod schedule of 14L:10D; the lights came on at 0800 GMT. Water and mixed seed were replenished on a daily basis. Millet (*Panicum miliacum*), which is the principal seed variety eaten by wild Zebra Finches (Morton and Davies 1983, Zann and Straw 1984), was provided once a week. Sources of dietary calcium (fine oystershell and cuttlefish bone), sand, and nest-building materials (hay, domestic hen feathers, and shredded paper) were provided *ad libitum*.

Clutch size ranged from two to seven eggs ($\bar{x}$ = 4.50 ± SD of 1.03, n = 84). The contents of nests were checked daily before 1300, by which time all eggs had been laid. Oviposition occurred between 0740 and 1225 ($\bar{x}$ = 0858 ± 56 min, n = 50). Eggs were removed from nest boxes of some females three days after the last egg was laid. This resulted in a period of follicular disruption and a cessation of egg laying in these control nonlaying females for at least the next five days (Haywood 1993).

Females that had produced a minimum of four eggs in previous laying attempts were selected for the egg-laying group of birds. Their laying period had to coincide with that of induced ovarian disruption in control birds. Egg-laying birds were allowed to lay three eggs on successive days to ensure that they were in a laying sequence. Nests were checked every 10 min on the morning the third egg was laid to determine the time of oviposition.

Administration of ^{45}Ca.—A dose of 5 μCi of radioisotopic calcium (^{45}Ca; ICN Flow Limited) in 0.1 ml distilled water was given by syringe to each female via a flexible silicone tube (<1 mm internal diameter) introduced into the proventriculus (see Houston et al. 1995b). An egg-laying female and a control bird were intubated as a pair exactly 1 h after the egg layer produced an egg.

Following dosing, birds were contained individually in cages (30 × 25.5 × 12 cm) equipped with a single perch and wall-mounted bowls for water and mixed seed. Clear perspex (DIY Plastics Limited) was sealed to the cage front with silicone rubber sealant, and a double layer of fine gauze was taped over the side opening of the cage to achieve full containment of radioactive aerosols. "Benchkote" trays were inserted into the bottom of cages to allow collection of feces during the bird's confinement. One pair of birds (egg layer and corresponding control) was sacrificed by CO_2 inhalation at 1, 2, 4, 10, 16, and 22 h post-intubation with ^{45}Ca. Birds were dissected and liver, heart, gizzard, gut, omentum, pectoral muscles, ovarian tissues, and skeleton (keel, humerus, radius, ulna, femur, fibula, and tibiotarsus; i.e. the bones containing principal medullary bone deposits) were removed for analysis.

Fecal collection.—Feces were collected from the floor trays in each cage at similar times to the above euthanasia schedule (i.e. 1, 2, 4, 10, 16, and 22 h post-intubation). To facilitate successive collections in the same cage and to minimize disturbance to the female, sheets of benchkote covered with paper towels were placed in the bottom of trays, the number of sheets corresponding to the number of collections required (e.g. for the 4-h birds, feces were collected three times at 1, 2, and 4 h [time of death] post-intubation). Feces were collected and the towel was in-

cluded in the sample for quantitation of radioactivity if it had been contaminated by feces.

Tissue preparation.—All tissue and fecal samples were weighed and distilled water was added in the ratio of 1 ml to 1 g wet tissue for every sample except the skeleton; 2.25 ml of 2M hydrochloric acid (i.e. an excess of acid) was added to each skeletal sample to obtain calcium in solution, leaving only the insoluble collagen matrix of the bone. All remaining tissue samples were coarsely chopped before NCS II tissue solubilizer (Amersham) was added at 48× (fecal samples) and at 24× (all other samples) the volume of the tissue-water homogenate. Suspensions were then incubated at 50°C for 24 h, or until they had cleared. Preparation of skeletal samples was complete after 24 h at room temperature.

Liquid-scintillation counting.—For all except skeletal samples, a 0.4-ml aliquot was added to 3.6 ml of OptiPhase Hisafe 3 scintillation fluid. A 0.1-ml aliquot of the skeletal digest was added to 4.9 ml of scintillant. All samples were prepared in duplicate and placed in darkness overnight to minimize the effects of chemiluminescence on the scintillation process. Samples were counted on a Beckman LS 1701 scintillation counter for 1 min. A standard curve was constructed from 2×10^{-2} to 9.8×10^{-6} μCi ^{45}Ca solutions. Radioactive background was calculated from appropriate tissue samples from undosed birds and subtracted from all experimental counts.

Statistical analyses.—Statistical analyses followed Sokal and Rohlf (1995). The effects of time after intubation of radioactive dose and of group (egg-laying or control females) on calcium content of various tissues were analyzed by two-way ANOVA (procedure ANOVA for balanced designs and procedure GLM for unbalanced designs; SAS Institute Inc. 1988). All calcium data were log transformed to reduce heteroscedasticity between samples. For all tissues except fecal samples, birds were placed into one of three groups based on time since intubation (0 to 8 h [1, 2, and 4 h], 8 to 16 h [10 h], and 16 to 24 h [16 and 22 h]) to provide suitable sample sizes for statistical comparisons. Sample sizes were sufficiently large for fecal analysis that the original collection times were retained for statistical comparisons. Comparisons between egg layers and controls within a time group were performed using one-tailed *t*-tests.

RESULTS

Calcium content of feces varied significantly with the amount of time elapsed since intubation of the radioactive dose (Table 1), with a peak in excretion, particularly in control birds, after 2 h (Fig. 1). Two hours after intubation, controls excreted significantly more calcium than did layers ($t = 2.15$, df = 16, $P < 0.025$).

TABLE 1. Results of two-way ANOVA comparing ^{45}Ca in the feces, skeleton, and ovarian tissues of egg-laying versus control Zebra Finches dissected at various times after administration of radioactive dose.

	df	*F*	*P*
Feces			
Time	5, 68	4.38	0.002
Group	1, 68	4.61	0.035
Time × group	5, 68	1.61	0.169
Skeleton			
Time	2, 44	0.07	0.936
Group	1, 44	16.94	0.0002
Time × group	2, 44	4.08	0.024
Ovaries			
Time	2, 46	5.09	0.010
Group	1, 46	6.83	0.012
Time × group	2, 46	5.71	0.006

Egg-laying birds retained significantly more of the administered calcium than did nonlaying control females (Table 1). Egg layers retained 2.5 times more calcium than controls during the first 4 h post-intubation; they excreted slightly more than 7% of the administered dose compared with nearly 18% excreted by controls. Controls excreted more than 20%, and layers nearly 13%, of the radioactive calcium in the 22 h following intubation.

Of the retained radioactive dose, up to 20% was incorporated into the skeletons of control and laying birds (Fig. 2). Layers incorporated significantly more calcium in skeletons than did controls during the first two time periods (0 to 8 h, $t = 4.31$, df = 44, $P < 0.0005$; 8 to 16 h, $t = 2.52$, df = 44, $P < 0.01$). There was a significant interaction of time × group for the incorporation of ^{45}Ca in skeletal tissue (Table 1). In the third time period (16 to 24 h), control birds incorporated more calcium than egg layers, but the difference was not significant ($t = 0.46$, df = 44, $P > 0.25$).

Calcium content of reproductive tissues varied significantly with time after dosing (Table 1). Egg-laying birds in the middle (8 to 16 h) period showed the most dramatic accumulation of calcium, having seven times more calcium in ovarian tissues than laying birds killed 0 to 8 h and 16 to 24 h after dosing (Fig. 3). Significantly more calcium was found in the ovarian tissues of egg-laying females compared with control birds (Table 1). Egg layers contained more than 60 times more calcium (0.25

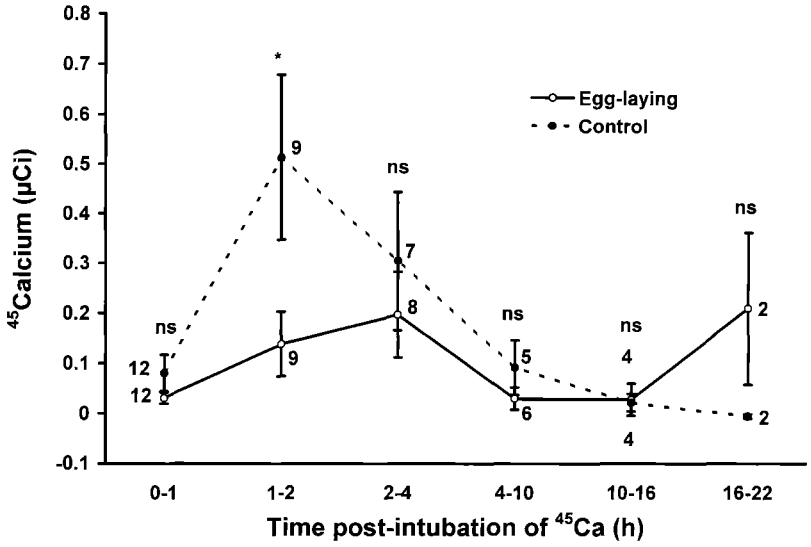

FIG. 1. ^{45}Ca content ($\bar{x} \pm 1$ SE) in feces for pairs of egg-laying and control Zebra Finches dosed with ^{45}Ca 1 h after oviposition in egg layers. Numbers represent sample sizes. Comparisons within time periods between egg layers and controls: ns, $P > 0.05$; *, $P < 0.025$.

$\pm$ 0.11 µCi) than did controls (0.004 $\pm$ 0.004 µCi; $t = 4.17$, df = 46, $P < 0.0005$; Fig. 3) 8 to 16 h after dosing. Egg-laying females in the other two time periods showed no significant differences in calcium incorporation compared with control females (0 to 8 h, $t = 0.23$, df = 46, $P > 0.25$; 8 to 16 h, $t = 0.92$, df = 46, $P > 0.10$).

Radioactive calcium content of liver tissue was analyzed to control for errors during dosing because no differences in hepatic calcium would be expected between egg-laying and control females. Calcium levels in livers declined significantly with time (Table 2), but egg-laying and control females had similar levels of hepatic calcium at equivalent times post-intubation (0 to 8 h, $t = 0.20$, df = 26, $P > 0.25$;

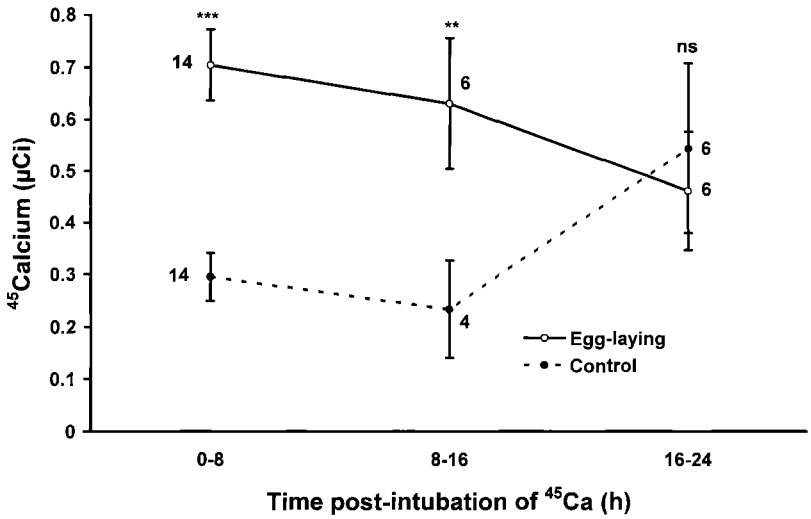

FIG. 2. ^{45}Ca content ($\bar{x} \pm 1$ SE) in skeletons of pairs of egg-laying and control Zebra Finches dosed with ^{45}Ca 1 h after oviposition in egg layers. Numbers represent sample sizes. Comparisons within time periods between egg layers and controls: ns, $P > 0.05$; **, $P < 0.01$; ***, $P < 0.0005$.

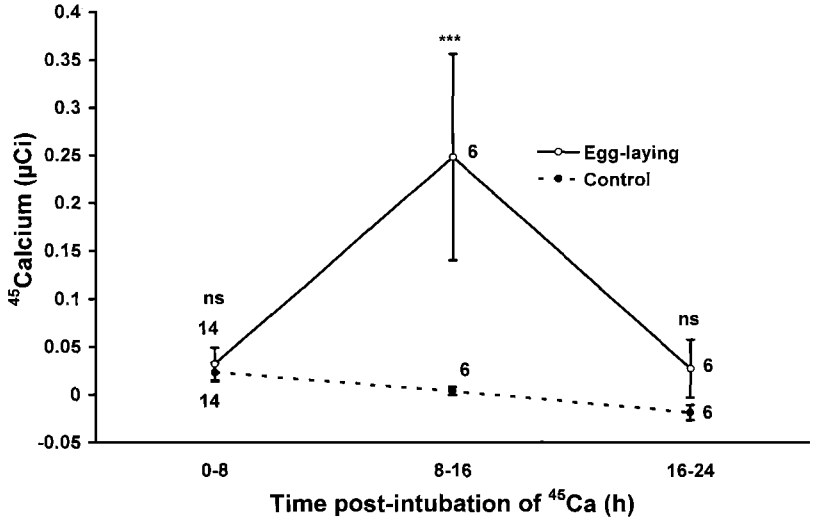

FIG. 3. ^{45}Ca content ($\bar{x} \pm 1$ SE) in ovarian tissues of pairs of egg-laying and control Zebra Finches dosed with ^{45}Ca 1 h after oviposition in egg layers. Numbers represent sample sizes. Comparisons within time periods between egg layers and controls: ns, $P > 0.05$; ***, $P < 0.0005$.

TABLE 2. ^{45}Ca content ($\bar{x} \pm$ SE, n in parentheses) in six tissues 0 to 8 h (Time = 1), 8 to 16 h (Time = 2), and 16 to 24 h (Time = 3) after radioisotope dosing of egg-laying and control Zebra Finches.

Time	Egg laying	Control
Gut ($F = 3.75$, df = 2 and 46, $P = 0.03$)[a]		
1	0.14 ± 0.05 (14)	0.60 ± 0.29 (14)
2	0.05 ± 0.02 (6)	0.01 ± 0.01 (6)
3	0.00 ± 0.01 (6)	0.00 ± 0.01 (6)
Gizzard ($F = 3.56$, df = 2 and 46, $P = 0.04$)		
1	0.12 ± 0.08 (14)	0.45 ± 0.19 (14)
2	0.00 ± 0.01 (6)	0.00 ± 0.01 (6)
3	0.02 ± 0.03 (6)	0.00 ± 0.01 (6)
Liver ($F = 5.61$, df = 2 and 46, $P = 0.007$)		
1	0.07 ± 0.02 (14)	0.08 ± 0.04 (14)
2	0.01 ± 0.01 (6)	0.00 ± 0.01 (6)
3	0.00 ± 0.01 (6)	−0.01 ± 0.01 (6)
Heart ($F = 5.56$, df = 2 and 46, $P = 0.007$)		
1	0.00 ± 0.00 (14)	0.01 ± 0.00 (14)
2	0.00 ± 0.01 (6)	0.00 ± 0.00 (6)
3	−0.01 ± 0.01 (6)	−0.01 ± 0.01 (6)
Muscle ($F = 2.81$, df = 2 and 46, $P = 0.07$)		
1	0.03 ± 0.03 (14)	0.01 ± 0.02 (14)
2	0.01 ± 0.03 (6)	0.00 ± 0.02 (6)
3	−0.05 ± 0.01 (6)	−0.03 ± 0.01 (6)
Omentum ($F = 2.68$, df = 2 and 44, $P = 0.08$)		
1	0.01 ± 0.00 (13)	0.05 ± 0.02 (13)
2	0.01 ± 0.00 (6)	0.01 ± 0.01 (5)
3	0.00 ± 0.00 (6)	0.00 ± 0.00 (6)

[a] Two-way ANOVA comparing ^{45}Ca content among time periods; all comparisons between groups and the interaction between time and group were not significant (all Ps > 0.10).

8 to 16 h, $t = 0.66$, df = 10, $P > 0.25$; 16 to 24 h, $t = 0.59$, df = 10, $P > 0.25$).

Radioactive calcium in the gut declined significantly with time post-dosing, especially in control birds killed at 0 to 8 h versus those sacrificed 8 to 16 h post-intubation (Table 2). Control birds had more calcium in their guts 0 to 8 h post-intubation than did egg-laying birds in the same period, but the difference was not significant ($t = 1.70$, df = 26, $P > 0.05$). No significant differences in gut retention of calcium occurred between egg layers and controls during the other two periods (8 to 16 h, $t = 1.65$, df = 10, $P > 0.05$; 16 to 24 h, $t = 1.35$, df = 10, $P > 0.10$).

Retention of calcium in the gizzard declined significantly with time post-dosing (Table 2). There were no significant differences in retention of calcium in the gizzard between egg-laying and control females at any time post-intubation (0 to 8 h, $t = 1.62$, df = 26, $P > 0.05$; 8 to 16 h, $t = 0.09$, df = 10, $P > 0.25$; 16 to 24 h, $t = 0.62$, df = 10, $P > 0.25$). Retention of calcium was very low in the three remaining tissues (heart, pectoral muscle, and omentum) in all birds regardless of whether they were egg layers or nonlayers. Less than 1% of the administered dose was found in any of these tissues. Measurement of nanocurie levels of radioactivity was subject to large counting errors (Table

2) and revealed inefficiencies in the liquid-scintillation counting process for detecting such low levels of radioactivity.

Discussion

Krementz and Ankney (1995) investigated changes in calcium with the onset of breeding in House Sparrows by measuring calcium content of ashed carcasses of birds obtained during prereproductive, prelaying, laying, postlaying, and incubating/brood-rearing periods (see Krementz and Ankney [1995] for definitions of categories). This holistic investigation revealed that total-body calcium levels increased prior to egg production, remained high until clutch completion, and declined after egg laying. I attempted to identify the mechanisms responsible for this elevated calcium retention during egg production. Furthermore, mine is the first study to use ^{45}Ca to quantify the partitioning of ingested calcium during egg laying in a small passerine. To date, this radioisotope has been used only in poultry as a tracer to study the calcium physiology of avian reproduction (e.g. Driggers and Comar 1949; Shirley et al. 1952; Tyler 1954; Hurwitz 1964, 1965; Mueller et al. 1964; Hurwitz et al. 1973; Farmer et al. 1986; Clunies et al. 1993).

Rapid incorporation of administered calcium into the skeletons of egg-laying birds occurred within the first 8 h after dosing (Fig. 2). Long bones were dissected selectively because they contain the principal deposits of medullary bone in prelaying females (Simkiss 1967). Similar to its role in poultry, medullary bone clearly represents a transitory and highly labile source of calcium during egg production in the Zebra Finch. Incorporation of calcium into the skeletons of egg layers remained significantly higher than in controls for the first 16 h of the ovulatory cycle, and declined to control levels only in the 16-to-24-h period thereafter. Medullary bone in small passerines is a short-term source of calcium (Krementz and Ankney 1995) that supplements the otherwise inadequate supply of calcium from the gut, satisfying the calcium requirements of the shell gland for complete calcification of the egg (Taylor 1970). Furthermore, calcium in the medullary bone appeared to have a diurnal pattern of repletion followed by depletion 0 to 8 h and 16 to 24 h into the ovulatory cycle, respectively. My findings support those of Krementz and Ankney (1995), who found that clutch size of House Sparrows was independent of endogenous calcium reserves because skeletal calcium was replenished from day to day rather than being depleted without replacement during the laying period.

Localization of calcium in the reproductive tissues of laying birds was highest at 8 to 16 h after intubation (Fig. 3). Calcification of the eggs of domestic hens does not start until approximately 9 h after ovulation (Taylor 1970, Farmer et al. 1986, Nys 1986). Timing of oogenetic events appeared to be similar in the Zebra Finch, with high levels of calcium present in the shell gland presumably when calcification started. Levels of ^{45}Ca in egg layers were similar to those in nonlayers 16 to 24 h after intubation, suggesting that calcification of the egg occurred predominantly in the middle period of the ovulatory cycle. In the domestic hen, shell secretion proceeds very slowly for the first 3 h that the egg is in the shell gland (Burmester 1940). Some workers have found that the rate of shell secretion after the first 3 h is linear until oviposition (Burmester 1940, Talbot and Tyler 1974), whereas others have found that shell secretion is most intense at 12 to 18 h post-oviposition (Clunies et al. 1993). My results support the findings of the latter study.

Calcium in the guts and gizzards of egg layers and nonlayers did not differ significantly at time of death (Table 2). However, nonlayers excreted more calcium in the first few hours post-dosing than did egg layers (Fig. 1); presumably, the layers retained more calcium during this period. Having depleted their calcium reserves for calcification of the previously laid egg, egg layers spent this period replenishing their deposits of medullary bone. Egg layers also might excrete less calcium overall compared with controls because the retention efficiency of the gut for dietary calcium increases during shell secretion (Hurwitz et al. 1973).

Clunies et al. (1993) split the ovulatory cycle of domestic hens into quarters and administered ^{45}Ca at the start of each (i.e. at 0, 6, 12, and 18 h post-oviposition). Intestinal and skeletal calcium dynamics were studied in detail for each 6-h period. Transfer of calcium between the digestive tract and the shell was lowest at 6 to 12 h post-oviposition, when calcium was preferentially deposited in bone. During the

early stages of egg calcification, calcium absorbed from the digestive tract was augmented by medullary bone reserves (as I have proposed for Zebra Finches). Highly productive females were in zero calcium balance over a single ovulatory cycle, with calcium absorption from the gastro-intestinal tract (1.716 g) almost equaling secretion as shell (1.704 g). Although my study provides no information about calcium balance in egg-laying Zebra Finches, I propose that a calcium balance of approximately zero must exist in egg-laying Zebra Finches to prevent depletion of calcium reserves (i.e. cortical bone) other than medullary deposits. Total-body calcium of House Sparrows was similar in birds captured on the last day of laying and those sacrificed in the post-laying period (Krementz and Ankney 1995).

In highly acidified areas where exogenous calcium is in limited supply, the incidence of eggshell defects and reduced clutch size in some bird populations has risen alarmingly in the last few years (e.g. Blancher and McNicol 1988, Ormerod et al. 1991, Graveland et al. 1994). My findings partly explain these phenomena; with limited calcium supplies, females go to roost with less calcium in their gizzards and less laid down as medullary bone (both sinks vital in supplying the overnight calcification of the egg). Calcium-specific foraging behavior during egg laying should be a rich area for future research efforts.

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