Comp. Biochem. Physiol. Vol. 113A, No. 4, pp. 343–349, 1996

ISSN 0300-9629/96/$15.00
SSDI 0300-9629(95)02074-8

Growth and Uptake of Mineral by Embryos of the Direct-Developing Frog *Eleutherodactylus coqui*

M.J. Packard,[1] *D.H. Jennings,*[2] *and J. Hanken*[2]

[1]Department of Biology, Colorado State University, Fort Collins, CO, USA;
[2]Department of Environmental, Population, and Organismal Biology, University of Colorado, Boulder, CO, USA

ABSTRACT. Embryos of the direct-developing frog *Eleutherodactylus coqui* take up small quantities of yolk and yolk mineral early in incubation but increase their uptake of yolk reserves at later stages of development. Growth and accumulation of calcium and magnesium by embryos also occur slowly at first and at a higher rate later. Accumulation of calcium and magnesium by embryos is largely a function of variation in size of embryos, but uptake of phosphorus is unrelated to size. Although patterns of growth and uptake of mineral by embryonic coquis resemble those for embryos of oviparous amniotes, embryonic coquis do not deplete the yolk of its nutrients to the same degree. Thus, residual yolk of coqui hatchlings contains a high percentage of the nutrient reserves originally present in the egg. This difference between embryonic coquis and embryos of oviparous amniotes may indicate that transfer of nutrients from yolk to embryo becomes limiting during the growth phase. Alternatively, some aspects of the neurologic system are so poorly developed at hatching that coqui may not be able to find prey effectively. A large nutrient reserve could sustain hatchlings while the neurologic system continues to mature. COMP BIOCHEM PHYSIOL 113A;4:343–349, 1996.

KEY WORDS. Anuran, calcium, direct development, embryo, frog, magnesium, mineral, phosphorus

INTRODUCTION

Eggs of direct-developing anuran amphibians are considerably larger than those of species with a larval stage and generally undergo a more protracted period of incubation (5,7,8). The large size of such eggs, coupled with the absence of a feeding larva, means that yolk must contain sufficient nutrients to support development of embryos that hatch as miniature versions of adults (7,8,33). In addition, eggs presumably must contain proportionately more calcium and phosphorus than would be predicted on the basis of large size alone because the evolution of direct development involved a significant change in the timing of ossification. This process occurs during embryogenesis in direct-developing species but occurs postembryonically in species with larvae that undergo metamorphosis (10,12–14,34, 36). In this regard, embryos of direct-developing anurans are similar to embryos of oviparous amniotes (10).

Precocious onset of ossification in direct-developing anurans has been noted in earlier studies (10), but patterns of uptake of calcium by embryos in these taxa have not been defined. These patterns are of particular interest because eggs of direct-developing amphibians may be similar in many respects to the evolutionary predecessor of the amniote egg (2–4,8). Thus, in addition to providing comparative information on patterns of vertebrate development, studies of embryos of direct-developing amphibians may provide insight into features of developmental physiology that underlie the evolution of the amniote egg. For these reasons, we measured the depletion of calcium, magnesium and phosphorus from yolk and its appearance in the carcass as a means of assessing uptake and deposition of mineral by embryos of the Puerto Rican direct-developing frog, *Eleutherodactylus coqui*. We also examined patterns of variation in dry mass of yolks and carcasses of embryos/hatchlings.

MATERIALS AND METHODS

Fertile eggs of *Eleutherodactylus coqui* were obtained from spontaneous matings among wild-caught adults maintained as a breeding colony at the University of Colorado. Eggs were placed on filter paper that had been moistened with deionized water and were incubated at 23.5°C in covered containers. Eggs were removed periodically from incubation and staged using standard criteria (33). Stages 1 and 2 and 5–14 (none of the eggs sampled was at stages 3 or 4) represent progressively more advanced stages of embryogenesis, whereas stage 15 represents newly emerged hatchlings (33). Individual eggs were placed into microcentrifuge tubes and stored frozen until the entire sample

Address reprint requests to: Mary J. Packard, Department of Biology, Colorado State University, Fort Collins, CO 80523 USA. E-mail: kpackard@holly.colostate.edu.

Received 25 April 1995; revised 4 August 1995; accepted 10 August 1995.

could be transferred to Colorado State University where they were thawed and dissected into components (jelly, yolk and embryo, depending on stage of development). Some eggs had to be dissected in a small drop of distilled water owing to desiccation prior to freezing.

Amphibian eggs are enclosed within thick layers of jelly at oviposiiton (1,6,29,31,37). We were unable to remove these layers at stages 1 and 2 and 9–14, but succeeded in removing the jelly from eggs at stages 5–8. Thus, we analyzed the jelly layers separately only for eggs at intermediate stages (*i.e.*, 5–8). Additionally, embryos at stages 1–8 were too small to be separated from yolk, so measurements prior to stage 9 combine carcass with yolk. Thereafter, embryos and yolks were analyzed separately.

Whole eggs or individual components (jelly, yolk or carcass) were weighed and dried to constant mass at 50°C to determine dry mass. Samples were placed in individual glass tubes and digested to clarity in a small quantity of nitric acid at 95°C. Digestates then were brought to volume in volumetric glassware. A 20-ml aliquot was taken from each volumetric flask and stored at 4°C in a capped plastic vial until analyses were performed for calcium, magnesium and phosphorus. Concentrations of calcium, magnesium, and phosphorus in digestates were determined using inductively coupled plasma atomic emissions spectrometry (32). Readings obtained using this procedure were converted to concentrations using a standard curve constructed from readings taken on a series of standard solutions containing appropriate quantities of calcium, magnesium and phosphorus. Data on concentration were used in conjunction with data on dry mass of eggs or components to estimate total quantities of calcium, magnesium and phosphorus in each sample.

None of the samples required dilution. Thus, none of the variation in the results is attributable to pipetting errors. Some variation results from small errors in weighing and digesting the samples and bringing them to volume. Some also is introduced by the atomic emissions spectrometer itself in that repeated readings of the same sample will not necessarily yield exactly the same value. We randomized all samples prior to analysis and made frequent readings of standards to distribute some of these potential sources of variation among the samples and to check for unusual variation in output. Experience has shown that variation attributable to such sources is negligible compared to variation among samples taken at different times during incubation.

Where appropriate, dry mass and mineral content of individual yolks were adjusted for the presence of jelly layers by subtracting the average dry mass or mineral content of jelly (as determined by analyses of jelly from eggs at stages 5–8). This manipulation provides more reasonable estimates of dry mass and mineral content of yolk alone. Carcasses are included in analysis of yolk at stages 1–8. However, dry mass and mineral content of carcasses at those stages is so low (see beyond) relative to yolk that the effect on dry mass or mineral content of yolk is essentially nil. Thus, yolk variables were not adjusted for the presence of the carcass at stages 1–8.

Data were analyzed statistically using mixed model analyses of variance and covariance with clutch as a random factor and stage as a fixed factor. Dry mass of carcasses or yolks was used as a potential covariate in analyses of covariance addressing the density of mineral in various fractions. The type III mean squares for the interaction was used as the error term in assessing effects of stage whereas that for residuals was used as the error term in assessing effects of clutch. A pooled estimate of the standard deviation is presented as a measure of dispersion in each analysis.

Eggs used in this study were taken from four different clutches, but each clutch is not represented at each stage of development. Clutch is a source of variation in many, but not all, analyses (Tables 1, 2 and 4). However, our focus will be on temporal patterns of change in dry mass and mineral content of yolks and carcasses, rather than on the contribution of clutch to patterns of variation.

RESULTS

Consumption of Yolk and Growth of Embryos

Stage of development contributes substantively to variation in dry mass of both yolks and carcasses (Table 1). Dry mass of yolks appears to decline fairly steadily during development (Fig. 1A), but this pattern may be more apparent than real (see Discussion). Dry mass of carcasses, on the other hand, is relatively small at stages 9 and 10, and then increases thereafter (Fig. 1B).

TABLE 1. Results of analyses of variance examining the effects of developmental stage and clutch on dry mass and Ca, Mg and P contents of carcasses and yolks of eggs of *Eleutherodactylus coqui*. Data for yolks are represented by stages 1–15; those for carcasses are represented by stages 9–15

	Source of variation	
Variable	**Stage**	**Clutch**
Dry mass of yolks	$F_{11,8} = 16.10$ $P < 0.001$	$F_{2,62} = 18.09$ $P < 0.001$
Dry mass of carcasses	$F_{6,3} = 61.65$ $P = 0.003$	$F_{2,34} = 2.81$ $P = 0.074$
Calcium in yolks	$F_{11,8} = 23.53$ $P < 0.001$	$F_{2,62} = 8.62$ $P < 0.001$
Magnesium in yolks	$F_{1,8} = 7.51$ $P = 0.004$	$F_{2,62} = 25.70$ $P < 0.001$
Phosphorus in yolks	$F_{11,8} = 8.37$ $P = 0.003$	$F_{2,62} = 7.24$ $P = 0.002$
Calcium in carcasses	$F_{6,3} = 19.95$ $P = 0.016$	$F_{2,34} = 4.57$ $P = 0.018$
Magnesium in carcasses	$F_{6,3} = 114.09$ $P = 0.001$	$F_{2,34} = 0.59$ $P = 0.560$
Phosphorus in carcasses	$F_{6,3} = 0.79$ $P = 0.633$	$F_{2,34} = 30.40$ $P < 0.001$

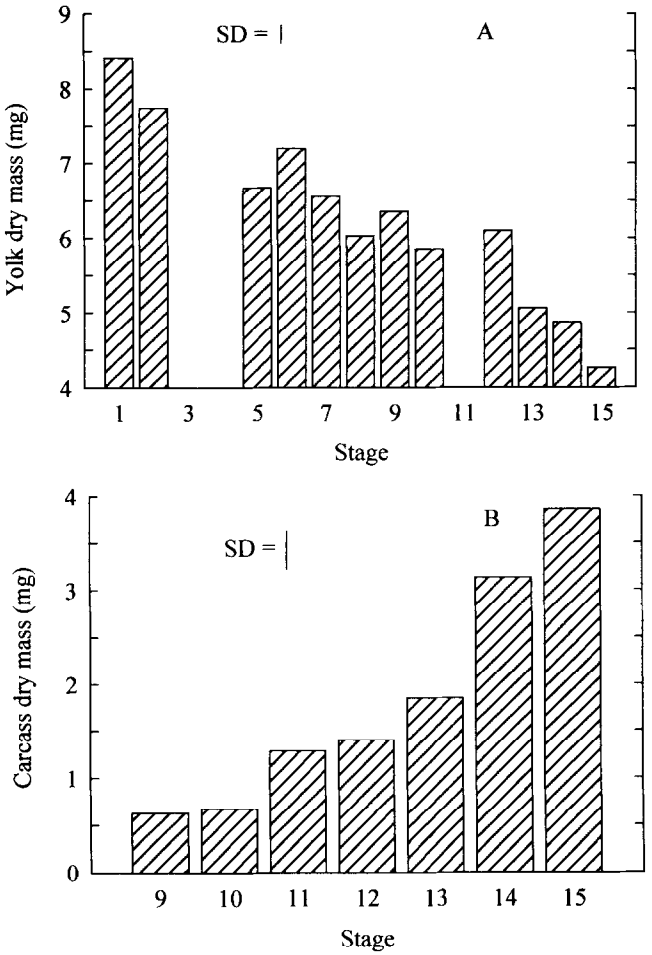

FIG. 1. (A) Mean values for dry mass (mg) of yolks removed from coqui eggs at stages 1–14 and from hatchlings at stage 15. Carcasses are included with yolks for eggs sampled at stages 1–8. (B) Means values for dry mass (mg) of yolk-free carcasses of coqui embryos (stages 9–14) and hatchlings (stage 15). Sample size varies from 4 to 11. The error bars represent pooled estimates of the standard deviation.

Mineral Content of Jelly Layers, Yolks and Carcasses

Stage and clutch of origin were minor sources of variation in dry mass and mineral content of the jelly layers (Table 2). Although stage contributed to variation in magnesium content, and clutch was a source of variation in dry mass and phosphorus content, overall variation in dry mass or mineral content of jelly is slight (Table 2). Jelly contains small but approximately equal quantities of calcium and phosphorus and trace amounts of magnesium (Table 3).

The pattern of change in mineral content of yolk is somewhat variable, perhaps owing to the combined effects of both stage and clutch of origin on the quantity of mineral in yolk (Table 1). However, mineral content of yolk generally declined more rapidly in later stages of development than in earlier stages (Fig. 2A, C and E). Calcium content of yolk declined from 39 to 13 μg between stage 1 and hatching; magnesium content declined from 20 to 10 μg; and phosphorus content from 103 to 53 μg (Fig. 2A, C and E).

TABLE 2. Results of analyses of variance examining the effects of developmental stage and clutch on dry mass and mineral content of jelly from eggs of *Eleutherodactylus coqui*

Variable	Source of variation: Stage	Source of variation: Clutch
Dry mass	$F_{3,2} = 7.86$ $P = 0.115$	$F_{2,13} = 5.25$ $P = 0.021$
Calcium	$F_{3,2} = 2.95$ $P = 0.263$	$F_{2,13} = 0.84$ $P = 0.453$
Magnesium	$F_{3,2} = 43.74$ $P = 0.028$	$F_{2,13} = 1.80$ $P = 0.205$
Phosphorus	$F_{3,2} = 1.18$ $P = 0.490$	$F_{2,13} = 4.72$ $P = 0.029$

Developmental stage is an important source of variation in calcium and magnesium content of coqui embryos/hatchlings, but does not affect phosphorus content (Table 1). Embryos accumulated relatively small quantities of calcium and magnesium in early stages of development, but the rate of accumulation increased as development proceeded (Fig. 2B and D). Thus, the pattern of variation in uptake of calcium and magnesium is qualitatively similar to the pattern of variation in dry mass of carcasses (Figs. 1B and 2B and D). Yolk-free carcasses of hatchlings contained 31.4 and 9.7 μg of calcium and magnesium, respectively (Fig. 2B and D). Deposition of phosphorus in carcasses does not resemble the patterns of variation in dry mass nor patterns of accumulation of calcium and magnesium (Figs. 1B and 2B, D and F). The quantity of phosphorus in carcasses ranged from 7 to 21 μg (Fig. 2E).

Data on calcium, magnesium and phosphorus in yolks and carcasses were subjected to analyses of covariance with dry mass of carcasses or yolks as the potential covariant to determine if carcasses and yolks at different stages of development exhibited different densities of mineral. The covariate was a significant source of variation in all analyses except that for phosphorus in carcasses (Table 4). Use of

TABLE 3. Average dry mass (mg) and mineral content (μg) of jelly layers of eggs of *Eleutherodactylus coqui* at early stages of development. Values in parentheses represent pooled estimates of the standard deviation

Stage	N	Dry mass	Ca	Mg	P
5	2	1.1	4.4	1.8	5.2
6	5	0.7	5.5	1.4	5.2
7	8	0.8	4.3	1.4	5.3
8	6	0.8	4.1	1.1	5.7
		(0.1)	(1.1)	(0.1)	(2.8)

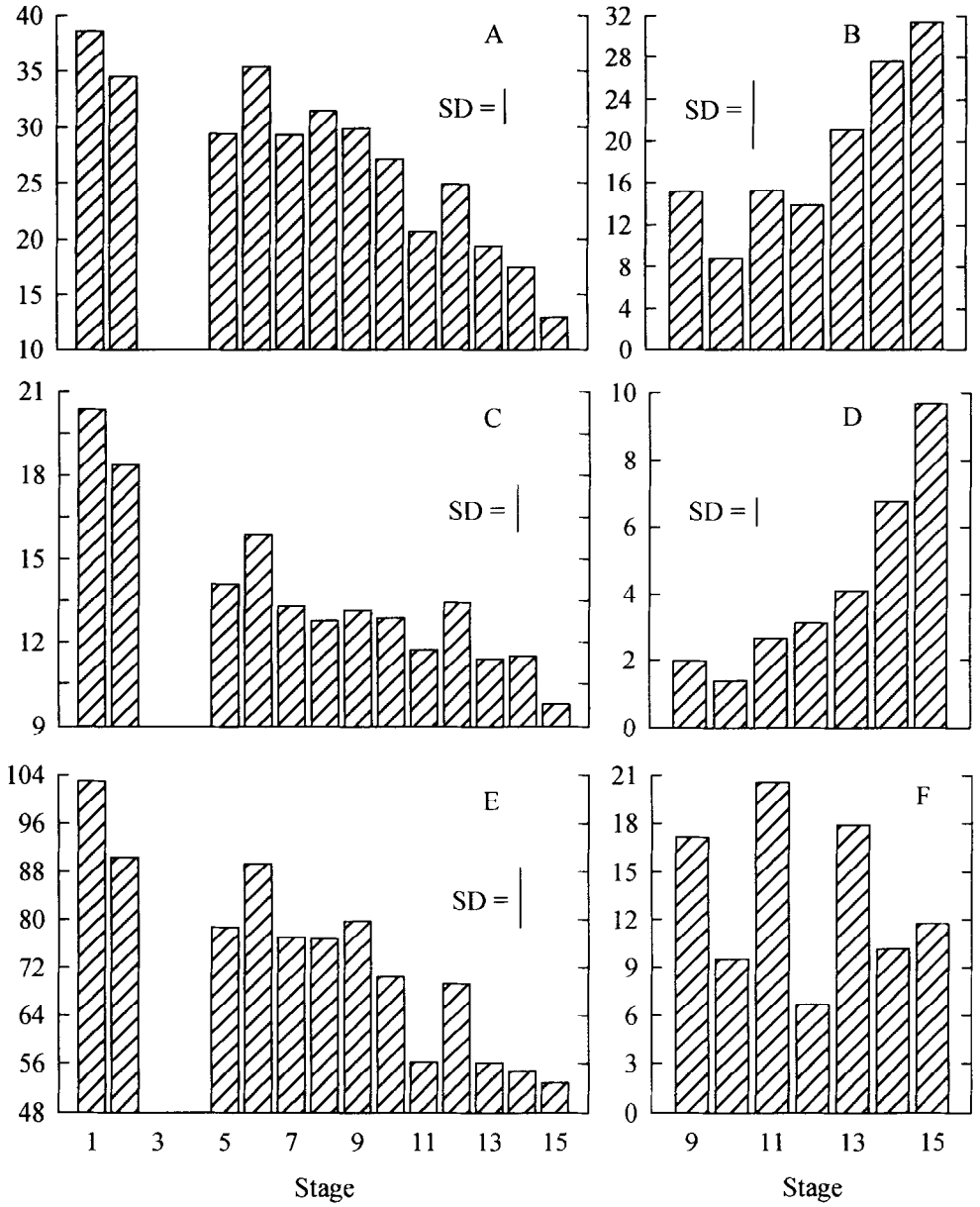

FIG. 2. Mean values for mineral content (μg) of yolks (stages 1–15) and yolk-free carcasses of coqui embryos (stages 9–14) and hatchlings (stage 15). (A) Calcium in yolks. (B) Calcium in carcasses. (C) Magnesium in yolks. (D) Magnesium in carcasses. (E) Phosphorus in yolks. (F) Phosphorus in carcasses. Sample size varies from 4 to 11. The error bars represent pooled estimates of the standard deviation. The standard deviation (17.1) for phosphorus in carcasses is too large to include on the graph.

the covariate effectively eliminated stage as a source of variation in the analyses (Tables 1 and 4), so the influence of stage on mineral content of carcasses and yolks is largely an effect of size. Larger embryos accumulate more mineral than smaller ones and thus deplete the yolk of more of its initial complement of mineral. When size is eliminated as a source of variation, carcasses and yolks from eggs at different stages contain similar quantities of mineral (data not shown). The analyses also indicate that size of embryos is not a factor in accumulation of phosphorus.

DISCUSSION

This is the first study of growth and uptake of mineral by embryos of an amphibian with direct development. Consequently, we cannot determine whether our findings are representative of direct-developing species in general or unique to *Eleutherodactylus coqui*. We will focus, therefore, on comparisons between coqui embryos and those of oviparous amniotes.

Embryonic development of oviparous amniotes typically can be divided into two phases: an initial period of differentiation when mobilization of yolk and growth are slow, and a later (growth) phase when nutrient uptake and growth are rapid (19,22,25,26). Thus, dry mass of yolk typically does not vary appreciably until embryos enter the growth phase, at which time dry mass begins to decline dramatically (19,22,25,26). Similarly, embryos exhibit relatively little change in dry mass during the differentiation phase but show substantial increase thereafter. In most respects,

TABLE 4. Results of analyses of covariance on Ca, Mg and P in yolks and carcasses of eggs of *Eleutherodactylus coqui*. Dry mass of yolks or carcasses was used as a potential covariate

	Source of variation		
Variable	**Covariate**	**Stage**	**Clutch**
Calcium in yolks	$F_{1,61} = 57.02$ $P < 0.001$	$F_{11,8} = 2.05$ $P = 0.158$	$F_{2,61} = 0.99$ $P = 0.378$
Magnesium in yolks	$F_{1,61} = 110.89$ $P < 0.001$	$F_{11,8} = 1.50$ $P = 0.287$	$F_{2,61} = 19.89$ $P < 0.001$
Phosphorus in yolks	$F_{1,61} = 104.55$ $P < 0.001$	$F_{11,8} = 0.83$ $P = 0.625$	$F_{2,61} = 8.41$ $P < 0.001$
Calcium in carcasses	$F_{1,33} = 13.31$ $P < 0.001$	$F_{6,3} = 1.14$ $P = 0.496$	$F_{2,33} = 7.49$ $P = 0.002$
Magnesium in carcasses	$F_{1,33} = 25.24$ $P < 0.001$	$F_{6,3} = 0.90$ $P = 0.586$	$F_{2,33} = 0.22$ $P = 0.804$
Phosphorus in carcasses	$F_{1,33} = 0.44$ $P = 0.513$	$F_{6,3} = 0.64$ $P = 0.707$	$F_{2,33} = 30.11$ $P < 0.001$

the pattern of variation in dry mass of yolks is the inverse of the pattern of variation in dry mass of carcasses (19,22,25,26). These patterns are unlikely to be unique to embryos of oviparous amniotes and would be expected to characterize most, if not all, species that undergo direct development and give rise to precocial young.

The pattern of variation in dry mass of yolks from coqui eggs does not exhibit relative stasis early in incubation followed by a dramatic decline later in development (Fig. 1A). Instead, dry mass seemingly declines more-or-less steadily throughout incubation. Yolk could undergo a physiologically meaningful decline in dry mass prior to stage 9 only if the costs of growth and maintenance during that period were extraordinarily high. We cannot eliminate this as an explanation for the apparent decline in dry mass prior to stage 9. Nonetheless, we view as unlikely the possibility that coqui embryos require an unusually high proportion of nutrient reserves as an energy source early in development.

More probable explanations have to do with variation in initial egg size (and thus variation in size and mineral content of yolk) and the fact that all clutches are not represented at each developmental stage. For example, eggs were not weighed at oviposition, so data cannot be adjusted for variation attributable solely to variation in size of eggs at the start of the study. In addition, eggs sampled at stages 1 and 2 are from the same clutch, eggs of that clutch were somewhat larger overall than eggs in other clutches, and this clutch is not represented at other stages. Thus, mean dry mass of eggs sampled early in incubation generally is higher than that of eggs sampled later, and this difference, coupled with the absence of samples at stages 3 and 4, contributes to the impression that dry mass declines steadily from stage 1 onward.

Classifying eggs on the basis of developmental stage, rather than day of incubation, also may underlie some of the variation in dry mass of yolks subsequent to stage 8. Even at a constant temperature, developmental rate may vary among embryos. As a result, nutrients may have been withdrawn from yolk at different rates despite the fact that embryos removed from those yolks were at the same developmental stage.

The pattern of variation in dry mass of carcasses is virtually identical to that reported for embryos of oviparous amniotes (19,22,25,26). Dry mass of carcasses, which we use as an index of growth, exhibits relatively little change at early stages followed by more substantial change later (Fig. 1B). Because eggs were classified by developmental stage instead of day of incubation, the midpoint of incubation and the temporal onset of the growth phase in coqui embryos cannot be identified with certainty. Nonetheless, the pattern of change in dry mass indicates that the growth phase probably begins when embryos are at stages 11–12 because that is when dry mass begins a period of relatively rapid increase (Fig. 1B). Indeed, a growth curve nearly identical to those described for a variety of embryos of oviparous amniotes could be superimposed onto the plot of dry mass of embryonic coqui (Fig. 1B) (19,22,25,26).

The jelly layers and yolk are the only probable sources of mineral available to coqui embryos. Although mineral content of jelly remains virtually constant during stages 5–8, we cannot be certain that mineral content of the jelly remains constant throughout incubation. Nonetheless, the jelly layers are unlikely to be an important source of mineral for embryos. Because amphibian eggs lack extraembryonic membranes, mineral in the jelly would have to diffuse through the jelly, cross the vitelline membrane, diffuse through the perivitelline space, and be absorbed by embryos. This mode of transfer also renders unlikely the absorption of mineral (or other nutrients) from the environment during development.

Patterns of variation in mineral content of yolks and carcasses of embryos of oviparous amniotes generally parallel patterns of variation in dry mass of yolks and carcasses: little change early in incubation followed by substantial change during the growth phase (18,21–24,26,27,30). Mineral content of yolks from coqui eggs certainly is reduced during development, but patterns of variation depart from expectations to varying degrees (Fig. 2A, C and D).

We did not sample perivitelline fluid in this study and thus cannot rule out the possibility that mineral is excreted or secreted into the perivitelline space during development. Nonetheless, patterns of variation in mineral content of yolks probably depart from expectations for the same reasons that dry mass does: variation in initial egg size; uneven distribution of clutches; and variation in age and developmental rate among embryos at the same developmental stage.

The pattern of accumulation of calcium and magnesium parallels the pattern of embryonic growth: small quantities of calcium and magnesium are deposited in carcasses early in development, but the rate of incorporation increases

as incubation proceeds. Accumulation of phosphorus in carcasses, on the other hand, does not increase as a function of developmental stage. Analyses of covariance reveal that the effect of stage on accumulation of calcium and magnesium is largely an effect of size, and we would expect size (*i.e.*, stage) to affect phosphorus accumulation in the same way. However, the strong variation attributable to clutch of origin indicates that clutch-specific responses may account for the anomalous results with respect to accumulation of phosphorus by coqui embryos.

The pattern of accumulation of calcium and magnesium generally parallels the pattern of ossification in coqui embryos (14). Ossification of the cranial skeleton is apparent by stage 12, and the postcranial skeleton stains intensely for calcium by stage 13 (14). Stage 12 precedes the period of accelerated deposition of calcium and magnesium in the carcass, whereas stage 13 marks the onset of this period (Fig. 2B and D).

Obvious endolymphatic deposits of calcium appear in coqui embryos during stage 6 and increase in size through stage 12 (33). After stage 12, the deposits decrease in size and are about one third of their original size at hatching. Further reduction in size occurs after hatching. These observations indicate that calcium removed from the yolk early in incubation may be deposited largely (if not exclusively) in the endolymphatic region. The regression of the deposits after stage 12 (33) indicates that embryos probably are redistributing calcium from the endolymphatics to the skeleton as they simultaneously absorb calcium from the yolk.

One potentially important difference between embryonic coqui and embryos of oviparous amniotes concerns the extent to which yolk is depleted of its nutrient reserves before hatching (18,21–24,26,27,30). Oviparous lizards and snakes typically reduce the mass of the yolk and its complement of mineral much more than is the case with coqui embryos. For example, embryonic bullsnakes (*Pituophis melanoleucus*) reduce yolk calcium by 97% and yolk phosphorus by 96% between oviposition and hatching (21); embryos of the bearded dragon (*Amphibolurus barbatus*) reduce dry mass of yolk and yolk calcium by 99% (28); and embryonic softshell turtles (*Trionyx spiniferus*) reduce dry mass of yolk by 90%, and yolk mineral is reduced by 95%, 96% and 95% for calcium, magnesium and phosphorus, respectively (23). The substantial reduction of yolk nutrients presumably allows embryos to attain near-maximal size prior to hatching.

The choice of initial values to use for similar calculations for coqui eggs is complicated by our concern that eggs sampled at stages 1 and 2 may be large compared to other eggs in our study. For this reason, we used a weighted average of dry mass and mineral content of eggs at stages 5–8 as the base from which calculations were made. These calculations revealed that dry mass of yolk of coqui eggs declines approximately 36% between stages 5–8 and hatching, whereas yolk mineral is reduced 59%, 31% and 35% for calcium, magnesium and phosphorus, respectively. These observations indicate that coqui embryos may hatch before they have achieved their full growth potential.

Several alternatives can be invoked to explain the observation that coqui embryos hatch with a considerably larger nutrient reserve in the yolk than is the case of embryos of oviparous amniotes. *Eleutherodactylus* eggs, like those of most other amphibians, undergo holoblastic cleavage during embryogenesis, and yolk is partitioned into individual cells as a result (8,10). A nutritional surface in the form of a vascularized yolk sac forms during development (8,15,16,35), but this epithelium presumably is in contact with individual yolk-filled cells rather than yolk itself. Eggs of oviparous amniotes undergo meroblastic cleavage, and most of the yolk remains uncleaved. As a result, the yolk sac is in intimate contact with yolk during much of incubation. The mechanism of nutrient uptake by the yolk sac of direct-developing amphibians has not been elucidated. Nonetheless, the absence of direct contact between the nutritional surface and the substrate may indicate that the transfer of nutrients from yolk to embryo becomes limiting during the growth phase. An inability to make greater use of the nutrient reserves of the yolk could constrain size of hatchlings even more than would be predicted on the basis of egg size alone (9).

On the other hand, it can be difficult to induce coqui hatched in captivity to feed even when prey of an appropriate size are offered (17). This difficulty may be an artifact of captivity but also may be related to maturity of the neurologic system. The retinotectal system of the eye and brain is so poorly developed at hatching that coqui may be unable to see or to see well enough to feed (G. Schlosser, personal communication). Under these circumstances, a large nutrient reserve in the form of residual yolk may sustain hatchlings until they are capable of feeding on their own.

We thank G.C. Packard for performing the statistical analyses and for constructive comments on drafts of this work. Supported in part by the NSF (IBN87-18191 and IBN94-07136 to M.J.P.; IBN93-21572 to D.H.J.; and DCB90-19624 and IBN94-19407 to J.H.).

References

1. Carroll, E.J., Jr.; Wei, S.H.; Nagel, G.M.; Ruibal, R. Structure and macromolecular composition of the egg and embryo jelly coats of the anuran *Lepidobatrachus laevis*. Devel. Growth Differ. 33:37–43;1991.
2. Carroll, R.L. Problems of the origin of reptiles. Biol. Rev. Cambridge Philos. Soc. 44:393–432;1969.
3. Carroll, R.L. Quantitative aspects of the amphibian–reptilian transition. Forma et Functio 3:165–178;1970.
4. Carroll, R.L. Vertebrate Paleontology and Evolution. New York: W. H. Freeman and Co.; 1988.
5. Duellman, W.E.; Trueb, L. Biology of Amphibians. New York: McGraw-Hill; 1986.
6. Dumont, J.N.; Brummett, A.R. Egg envelopes in vertebrates. In: Browder, L.W., ed. Developmental Biology, Vol. 1. New York: Plenum Press; 1985: 235–288.

7. Elinson, R.P. Fertilization and aqueous development of the Puerto Rican terrestrial-breeding frog, *Eleutherodactylus coqui*. J. Morphol. 193:217–224;1987.
8. Elinson, R.P. Change in developmental patterns: Embryos of amphibians with large eggs. In: Raff, R.A.; Raff, E.C., eds. Development as an Evolutionary Process. New York: Alan R. Liss; 1987: 1–21.
9. Elinson, R.P. Egg evolution. In: Wake, D.B.; Roth, G., eds. Complex Organismal Functions: Integration and Evolution in Veterbrates. Chichester, UK: Wiley; 1989: 251–262.
10. Elinson, R.P. Direct development in frogs: Wiping the recapitulationist slate clean. Sem. Dev. Biol. 1:263–270;1990.
11. Elinson, R.P.; de Pino, E.M.; Townsend, D.S.; Cuesta, F.C.; Eichhorn, P. A practical guide to the developmental biology of terrestrial-breeding frogs. Biol. Bull. Woods Hole 179:163–177;1990.
12. Hanken, J.; Hall, B.K. Variation and timing of the cranial ossification sequence of the Oriental fire-bellied toad, *Bombina orientalis* (Amphibia, Discoglossidae). J. Morphol. 182: 245–255;1984.
13. Hanken, J.; Hall, B.K. Skull development during metamorphosis: I. Early development of the first three bones to form—the exoccipital, the parasphenoid, and the frontoparietal. J. Morphol. 195:247–256;1988.
14. Hanken, J.; Klymkowsky, M.W.; Summers, C.H.; Seufert, D.W.; Ingebrigtsen, N. Cranial ontogeny in the direct-developing frog, *Eleutherodactylus coqui* (Anura: Leptodactylidae), analyzed using whole-mount immunohistochemistry. J. Morphol. 211:95–118;1992.
15. Lynn, W.G. The embryology of *Eleutherodactylus nubicola*, an anuran which has no tadpole stage. Contrib. Embryol. Carnegie Inst. Wash. (Publ. 541) 30:27–62;1942.
16. Lynn, W.G.; Lutz, B. The development of *Eleutherodactylus guentheri* Stdnr. 1864. Bol. Mus. Nac. Zool. 71:1–46;1946.
17. Michael, S.F. Captive breeding of two species of *Eleutherodactylus* (Anura: Leptodactylidae) from Puerto Rico, with notes on behavior in captivity. Herpetol. Rev. 26:27–29; 1995.
18. Miller, J.D.; Jones, M.E. Growth and calcium metabolism of embryos of the long-necked tortoise, *Chelodina longicollis* (Shaw). Mem. Queensland Mus. 29:421–436;1990.
19. Morris, K.A.; Packard, G.C.; Boardman, T.J.; Paukstis, G.L.; Packard, M.J. Effect of the hydric environment on growth of embryonic snapping turtles (*Chelydra serpentina*). Herpetologica 39:272–285;1983.
20. Packard, M.J.; Packard, G.C. Effect of water balance on growth and calcium mobilization of embryonic painted turtles (*Chrysemys picta*). Physiol. Zool. 59:398–405;1986.
21. Packard, M.J.; Packard, G.C. Sources of calcium and phosphorus during embryogenesis in bullsnakes (*Pituophis melanoleucus*). J. Exp. Zool. 246:132–138;1988.
22. Packard, M.J.; Packard, G.C. Mobilization of calcium, phosphorus, and magnesium by embryonic alligators, *Alligator mississippiensis*. Am. J. Physiol. 257:R1541–R1547;1989.
23. Packard, M.J.; Packard, G.C. Sources of calcium, magnesium, and phosphorus for embryonic softshell turtles (*Trionyx spiniferus*). J. Exp. Zool. 258:151–157;1991.
24. Packard, M.J.; Packard, G.C. Patterns of mobilization of calcium, magnesium, and phosphorus by embryonic yellow-headed blackbirds (*Xanthocephalus xanthocephalus*). J. Comp. Physiol. B. 160:649–654;1991.
25. Packard, G.C.; Packard, M.J.; Boardman, T.J.; Morris, K.A.; Shuman, R.D. Influence of water exchanges by flexible-shelled eggs of painted turtles *Chrysemys picta* on metabolism and growth of embryos. Physiol. Zool. 56:217–230; 1983.
26. Packard, M.J.; Packard, G.C.; Gutzke, W.H.N. Calcium metabolism in embryos of the oviparous snake *Coluber constrictor*. J. Exp. Biol. 110:99–112;1984.
27. Packard, M.J.; Short, T.M.; Packard, G.C.; Gorell, T.A. Sources of calcium for embryonic development in eggs of the snapping turtle *Chelydra serpentina*. J. Exp. Zool. 230:81–87; 1984.
28. Packard, M.J.; Packard, G.C.; Miller, J.D.; Jones, M.E.; Gutzke, W.H.N. Calcium mobilization, water balance, and growth in embryos of the agamid lizard *Amphibolurus barbatus*. J. Exp. Zool. 235:349–357;1985.
29. Salthe, S.N. The egg capsules in the Amphibia. J. Morphol. 113:161–171;1963.
30. Shadrix, C.A.; Crotzer, D.R.; McKinney, S.L.; Stewart, J.R. Embryonic growth and calcium metabolism of oviposited eggs of the scincid lizard, *Eumeces fasciatus*. Copeia 493–498; 1994.
31. Shivers, C.A.; James, J.M. Morphology and histochemistry of the oviduct and egg-jelly layers in the frog, *Rana pipiens*. Anat. Rec. 166:541–556;1970.
32. Soltanpour, P.N.; Jones, J.B., Jr.; Workman, S.M. Optical emission spectrometry. In: Page, A.L., ed. Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties, 2nd ed. Agronomy Monograph 9. Madison, WI: American Society of Agronomy and Soil Science Society of America; 1982: 29–65.
33. Townsend, D.S.; Stewart, M.M. Direct development in *Eleutherodactylus coqui* (Anura: Leptodactylidae): A staging table. Copeia 423–436;1985.
34. Trueb, L. A summary of osteocranial development in anurans with notes on the sequence of cranial ossification in *Rhinophrynus dorsalis* (Anura: Pipoidea: Rhinophrynidea). S. Afr. J. Sci. 81:181–185;1985.
35. Valett, B.B.; Jameson, D.L. The embryology of *Eleutherodactylus augusti latrans*. Copeia 103–109;1961.
36. Wiens, J.J. Ontogeny of the skeleton of *Spea bombifrons* (Anura: Pelobatidae). J. Morphol. 202:29–51;1989.
37. Yurewicz, E.C.; Oliphant, G.; Hedrick, J.L. The macromolecular composition of *Xenopus laevis* egg jelly coat. Biochemistry 14:3101–3107;1975.