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Mechanistic Basis of Life History Evolution in Anuran Amphibians: Thyroid Gland Development in the Direct-Developing Frog, *Eleutherodactylus coqui*

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Direct development is a widespread, alternative life history in Recent amphibians. There is no free-living, aquatic larva; adult features form in the embryo and are present at hatching. The mechanistic bases of direct development remain relatively unexplored. The current study describes the embryonic ontogeny of the thyroid gland in the direct-developing frog *Eleutherodactylus coqui* (Leptodactylidae) and quantifies histological changes that occur in the gland after its initial appearance. The thyroid gland of *E. coqui* is first apparent at Townsend-Stewart stage 10, approximately two-thirds of the way through embryogenesis. Soon after this the thyroid begins to accumulate follicular colloid. Quantitative analyses of thyroid histology reveal embryonic peaks in two measures, follicle number and follicle volume, which are followed by declines in these measures prior to hatching. These peaks in thyroid activity in *E. coqui* are correlated with morphological changes that are directly comparable to metamorphic changes in frogs that retain the ancestral, biphasic life history. In metamorphic taxa, a histologically identifiable thyroid gland does not form until the larval period, well after hatching. Nevertheless, measures of thyroid histology observed in *E. coqui* follow the pattern reported for metamorphosing amphibians. The present results support the hypothesis that the evolution of direct development in anurans is associated with precocious development and activity of the thyroid axis.

Complex life cycles, with distinct larval and adult stages separated by a period of rapid morphological remodeling, or metamorphosis, are among the most common developmental strategies in animals (Moran, 1994; Davidson *et al.*, 1995). Metamorphosis is generally considered a diagnostic feature of amphibian development. However, many species of amphibians exhibit alternative life history strategies that involve significant modification, or even elimination, of a posthatching metamorphosis (Duellman and Trueb, 1986). Among the most extreme of these alternative life histories is direct development, which is characterized by absence of the free-living, aquatic larval stage and the direct, embryonic formation of adult features. Direct development is a widespread reproductive mode in Recent amphibians and especially frogs. It has evolved independently one or more times within each of the three extant orders—frogs, salamanders, and caecilians—and characterizes many hundreds of living species (Wake, 1989). Indeed, it is the predominant reproductive mode in some clades, e.g., plethodontid salamanders (Wake and Hanken, 1996). Direct development provides excellent opportunities to examine the mechanistic bases of life history evolution as well as their morphological and ecological consequences (Hanken, 1992; Hanken *et al.*, 1997a). Yet the

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mechanistic bases of direct development remain poorly known (Elinson, 1994; Wake and Hanken, 1996).

In metamorphosing amphibians, the transition from aquatic larva to terrestrial adult is under complex hormonal control. Although a number of hormones are involved in mediating metamorphic transitions, most analyses of endocrine regulation of metamorphosis have focused on the thyroid axis (Norris and Dent, 1989; Kikuyama *et al.*, 1993; Denver, 1996; Hayes, 1997). Since species with direct development bypass the larval stage and generate the adult, postmetamorphic morphology directly, a number of authors have identified two kinds of change in the ancestral hormonal mediation of metamorphosis that could underlie the evolution of direct development (Lynn, 1936, 1942; Dent, 1942; Lynn and Peadon, 1955). The first possible change is early, embryonic activation of the same hormonal axes that control metamorphosis in ancestral amphibians, which could result in the precocious formation of adult features in direct developers. Alternatively, tissues dependent on hormonal regulation in metamorphosing taxa may have become freed from hormonal control in direct developers.

Few studies have examined the endocrine control of direct development (Hanken *et al.*, 1997a). Most of these analyses have relied on hormonal manipulations, which employ exogenous thyroid hormone (TH) or TH antagonists (Lynn, 1948; Lynn and Peadon, 1955; Hughes, 1966; Hughes and Reier, 1972; Elinson, 1994; Callery and Elinson, 1996). Such manipulations of TH levels in direct-developing amphibians have shown that some features of development are still under TH control, while others seem to have been emancipated from the requirement for TH. Although suggestive, these results must be interpreted with caution as emancipation from TH regulation is only one possible explanation for the inability of exogenous TH to accelerate development. Exogenous TH may have no effect on morphogenesis because tissues are maximally stimulated by endogenous TH (Elinson, 1994), because hormone treatments alter the actions of TH synergists (Kikuyama *et al.*, 1993), or because TH receptors are not expressed at the stage(s) examined. In addition, treatment with thyroid inhibitors (thiourea and phenylthiourea) may cause pharmacological effects that are not comparable to normal development. A complete understanding of the role of TH in direct development requires more detailed knowledge about the normal differentiation and function of the thyroid axis.

This study describes the embryonic ontogeny of the thyroid gland in the direct-developing frog *Eleutherodactylus coqui* (Leptodactylidae) and quantifies histological changes that occur in the gland after its initial appearance. Since the thyroid, unlike most other endocrine glands, stores the hormone it produces extracellularly (in follicles), changes in thyroid histology have been widely used to assess TH production during development and metamorphosis. Numerous studies of thyroid ontogeny and activity in metamorphosing amphibians have documented a correlation between thyroid activity and the length of the larval period (Iwasawa, 1966; Francois-Krassowska, 1978, 1982, 1989). Among the more frequently examined histological features used to measure thyroid activity in metamorphosing taxa are gland volume, follicle volume, number of colloid-filled follicles, and epithelial cell height. Although brief descriptions of thyroid gland development are available for several species of *Eleutherodactylus* (Lynn 1936, 1942, 1948; Lynn and Lutz 1946; Lynn and Peadon, 1955), no detailed analysis of thyroid gland ontogeny and activity is available for any amphibian with direct development. If direct development in *E. coqui* is mediated by precocious thyroid activity, the thyroid gland should be histologically identifiable during embryogenesis and should exhibit changes that are correlated with the formation of adult features.

MATERIALS AND METHODS

Animal Care

Adult *E. coqui* were collected with the permission of the Puerto Rico Department of Natural Resources (permits DRN-91–45, DRN-92–19, DRN-93–26, and DRNA-95–26). Developmental series of embryos were obtained from spontaneous matings of wild-caught animals maintained as a laboratory breeding colony at the University of Colorado. Fertilized eggs were cultured in petri dishes lined with filter paper moistened with 10% Holtfreter's solution. Petri dishes were covered and placed in a darkened incubator at 25°C.

Staging and Samples

Embryos were staged according to the table of Townsend and Stewart (TS; 1985), which is specific for *E. coqui*. Development is divided into 15 embryonic stages, from oviposition (TS 1) to hatching (TS 15). Length of embryonic development of *E. coqui* at 25°C is 16.8 days (Townsend and Stewart, 1986). Samples consisted of four embryos per stage; each sample included embryos from at least two unrelated clutches.

Histology

Embryos were anesthetized in 0.03% tricaine-methanesulfonate (Sigma Chemical Co., St. Louis, MO), fixed in 10% neutral-buffered formalin, dehydrated in ethanol, and embedded in Paraplast. Serial sections (10 μm) of entire embryos were prepared and stained with a four-part connective tissue stain (Alcian blue, direct red, celestine blue and hematoxylin; Hall, 1985).

Measurements

Thyroid measurements were taken using an Olympus Cue-2 image analysis system connected to a Leitz compound microscope. Measurements were made for four embryos at each stage between the initial appearance of the gland (TS 10) and hatching (TS 15). The following measures were taken for all sections of both left and right thyroid glands in each embryo: gland area, area of each follicle, colloid area within each follicle, and epithelial cell height at four randomly chosen points along each follicle. Follicle number was quantified by counting the number of follicles present in the largest section in each lobe. Volume measurements (gland volume, follicle volume, colloid volume) were calculated by multiplying summed area measurements by section thickness. Percentage of colloid was calculated by dividing colloid volume by gland volume for each specimen. Mean epithelial cell height for each specimen was calculated by dividing summed epithelial cell height measurements by the total number of epithelial measurements made on that specimen. Data in the text are presented as means ± one standard error of the mean.

Statistics

A Tukey-HSD analysis of variance was used to assess stage-related changes in individual thyroid measures. Pearson correlation coefficients were used to examine the relationship among thyroid measures during development. All analyses were performed using SPSS.

RESULTS

Thyroid Histology

The thyroid is first apparent at TS stage 10, approximately two-thirds of the way through embryogenesis (hatching occurs at stage 15). At this stage, the glands comprise a pair of distinct masses ventral and lateral to the posterior portion of the hyobranchial skeleton. Each gland extends posterior to the hyobranchial skeleton and terminates dorsolateral to the anterior margin of the developing heart. Follicles are poorly organized, and no colloid is present (Fig. 1A). By stage 11 the glands are organized into follicles, each containing a central lumen. Most lumina are beginning to accumulate colloid, although one specimen showed no sign of colloid formation. All specimens at stage 12 have thyroids with well-developed follicles containing colloid, and several specimens contain vacuoles within the colloid. Thyroid histology changes little throughout the remainder of embryogenesis (stages 13–15); colloid-filled follicles, some of which contain vacuoles, are present in all specimens (Fig. 1B).

Quantification of Thyroid Histology

Overall gland volume decreases slightly, from 4944 ± 871 to 3477 ± 380 μm^3, between the initial appearance of the gland at stage 11 and hatching (Fig. 2A). There is no significant difference in gland volume among any of the stages. Follicle number, follicle volume, colloid volume, and percentage of colloid are highest at stage 13 and then decrease prior to hatching (Figs. 2B–2E). Follicle number increases from 6.5 ± 3.12 (TS 11) to 19.5 ± 1.71 (TS 13) and then decreases to 11 ± 2.04 at hatching (TS 15). Total follicle volume increases from an initial value of 635 ± 185 μm^3 (TS 11), peaks at

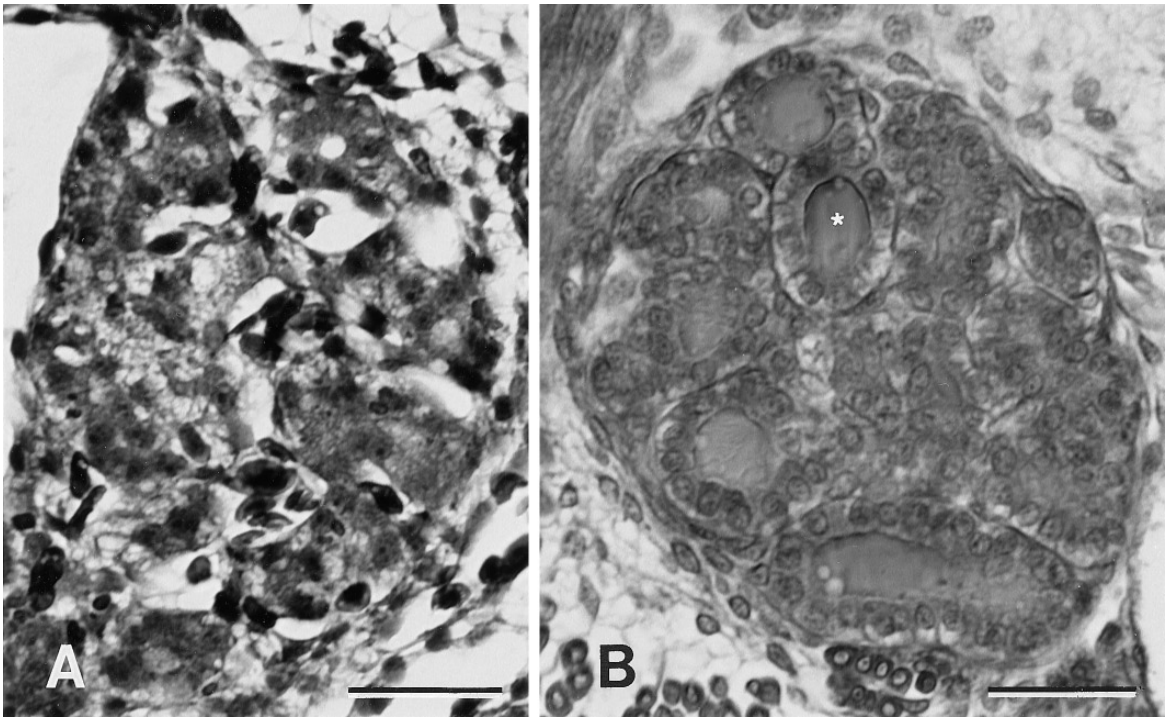

FIG. 1. Thyroid gland histology of *E. coqui*. (A) TS stage 10. (B) Stage 13. Several colloid-filled follicles are visible (asterisk). Transverse sections, dorsal is at the top. Bars, 20 μm.

1488 ± 316 μm^3 (TS 13), and then decreases to 846 ± 161 μm^3 (TS 15). Significant differences were found in follicle number between stages 11 and 13 ($F = 2.61$, $P < 0.05$) and in follicle volume between stages 12 and 13 ($F = 3.28$, $P < 0.05$). Colloid volume rises from an initial value of 192 ± 97 μm^3 (TS 11) to a peak of 594 ± 192 μm^3 (TS 13), before decreasing to 275 ± 50 μm^3 (TS 15). Percentage of colloid is 3.6 ± 2.29% at stage 11, rises to 15.1 ± 3.98% at stage 13, and declines to 8.3 ± 1.92% at stage 15. No significant difference in either colloid volume or percentage of colloid was found among stages. Follicular epithelial cell height gradually decreases from 568 ± 60 to 458 ± 61 μm throughout embryonic development (Fig. 2F). This decrease is not statistically significant.

Correlations among thyroid measures between stages 11 and 15 are given in Table 1. Gland volume was not significantly correlated with any other measure of thyroid histology. Follicle volume was significantly correlated with both colloid volume ($r = 0.965$, $P < 0.01$) and follicle number ($r = 0.693$, $P < 0.01$). Colloid volume and epithelial cell height were also significantly correlated with follicle number ($r = 0.634$, $P < 0.01$, and $r = -0.599$, $P < 0.01$, respectively).

DISCUSSION

Ontogenetic Patterns

The genus *Eleutherodactylus* has been the focus of most studies of TH control of direct development in amphibians, yet thyroid ontogeny has never been comprehensively described for any species. Several previous studies report the thyroid gland present during late stages of embryogenesis in various species (*E. nubicola*, Lynn 1936, 1942; *E. guentheri*, Lynn and Lutz 1946; *E. ricordii*, Lynn 1948; *E. martinicensis*, Lynn and Peadon, 1955). However, none of these analyses was based on a complete developmental series. The current study of *E. coqui* is the first to document the complete time course of thyroid gland ontogeny in any species of *Eleutherodactylus*. In *E. coqui*, the thyroid gland first appears at TS stage 10, approximately

two-thirds of the way through embryogenesis. Thyroid follicles begin to accumulate colloid shortly thereafter (TS 11). This observation is consistent with the limited data available for other species of *Eleutherodactylus*, which document initial appearance of the thyroid and accumulation of thyroid colloid during later stages of embryogenesis (Lynn, 1936, 1942; Dent, 1942; Lynn and Peadon, 1955). Thyroid differentiation in direct-developing salamanders also occurs prior to hatching, during embryogenesis (Dent, 1942, 1954).

Onset of thyroid development during embryogenesis in direct-developing amphibians differs markedly from the timing of thyroid development in metamorphosing taxa. Thyroid differentiation has been described for many species of metamorphic frogs, including *Bufo japonicus*, *Rana tagoi*, *R. ornatoventris*, *R. catesbeiana*, *Rhacophorus arboreus* (Iwasawa, 1966), *Bombina bombina*, *B. variegata*, *Pelobates fuscus*, *Hyla arborea*, *Rana temporaria* (Francois-Krassowska, 1978), *Bufo calamita* (Francois-Krassowska, 1989), and *Xenopus laevis* (Nieuwkoop and Faber, 1956; Saxen *et al.*, 1957). In all these species, the thyroid differentiates during the larval period, well after hatching. Embryonic onset of thyroid development in *E. coqui* is consistent with the hypothesis that the evolution of direct development in anuran amphibians involved precocious activation of the thyroid axis (Lynn, 1936; Dent, 1942; Lynn and Peadon, 1955).

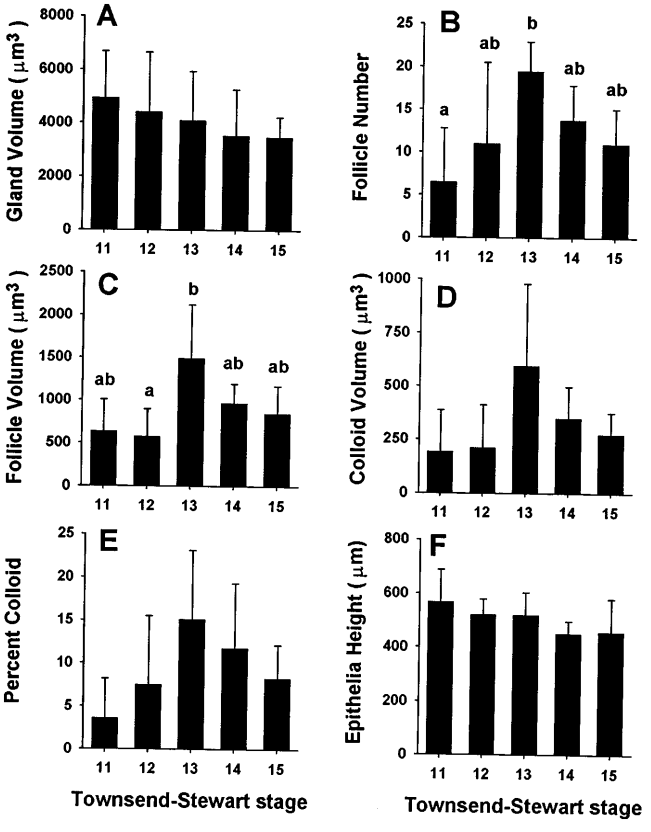

FIG. 2. Measurements of several parameters of thyroid activity in *E. coqui*. Each bar represents the mean of four specimens ± 2 SEM. Groups with no superscripts and groups sharing superscripts are not significantly different (Tukey-HSD; $P > 0.05$). (A) Gland volume, (B) follicle number, (C) follicle volume, (D) colloid volume, (E) percentage of colloid, and (F) epithelial cell height.

TABLE 1
Pearson Correlation Coefficients among Five Measures of Thyroid Histology during Late Stages of Embryogenesis (TS 11-15) in *Eleutherodactylus coqui*

	Follicle volume	Colloid volume	Epithelial cell height	Follicle number
Gland volume	−0.084	−0.096	−0.210	0.197
Follicle volume		0.965*	−0.169	0.693*
Colloid volume			−0.273	0.634*
Epithelial cell height				−0.599*

* Significant at $P < 0.01$.

Quantitative Comparisons

Overall gland volume of the thyroid in *E. coqui* appears to decrease during embryogenesis, although differences among stages are not significant (Fig. 2A). This pattern differs from that reported for metamorphosing taxa, in which overall gland volume increases during prometamorphosis and then declines prior to the completion of metamorphosis (Iwasawa, 1966; Francois-Krassowska, 1978, 1982, 1989). One likely explanation for the different temporal patterns of gland volume between *E. coqui* and metamorphosing frogs is that *E. coqui* does not grow substantially during the latter portion of the embryonic period when the gland develops. In contrast, metamorphosing amphibians grow substantially between hatching and the onset of metamorphic climax.

A second possible explanation for the differing patterns of change in gland volume between *E. coqui* and metamorphosing frogs is that changes in gland volume could result from changes in connective tissue or vascular components of the gland. In the metamor-

phosing frog *Bufo angusticeps* blood supply increases during metamorphic climax, whereas in *Arthroleptella villiersi*, a frog that passes through a modified, nonfeeding larval stage prior to metamorphosis, blood supply is highest during early stages of metamorphosis and declines only after the completion of metamorphosis (Brink, 1939). Changes in overall thyroid gland volume in *E. coqui* may reflect changes in the vascular component of the gland, rather than alterations in the rate or amount of hormone production.

The lack of correlation between gland volume and other thyroid measures (Table 1) potentially indicates that these other measures (i.e., follicle number, follicle volume, and colloid volume) may provide more accurate measures of thyroid activity than does gland volume. Follicle number and follicle volume both peak during late embryogenesis (TS 13) and then decline prior to hatching (Figs. 2B and 2C). Colloid volume does not change significantly from the initial appearance of colloid at stage 11 until hatching at stage 15 (Fig. 2D). The general lack of significant differences in thyroid measurements among stages has also been reported for metamorphosing frogs (Etkin, 1936; D'Angelo and Charipper, 1939; Francois-Krassowska, 1978, 1989). It likely is the result of high levels of interindividual variation within a stage and the lack of precision of staging tables (Francois-Krassowska, 1978).

Changes in epithelial cell height of thyroid follicles are among the most frequently reported measures of thyroid activity. Because epithelial cell height is regulated by thyroid-stimulating hormone (TSH) produced by the pituitary, it is widely used as an indicator of thyroid stimulation by TSH (Norris, 1997). In metamorphosing amphibians, epithelial cell height increases during early metamorphosis and then declines prior to the completion of metamorphic climax (D'Angelo and Charipper, 1939; Iwasawa, 1966; Francois-Krassowska, 1978, 1982, 1989). In contrast, epithelial cell height in *E. coqui* does not change significantly following the initial appearance of colloid at stage 11 until hatching (Fig. 2F). This pattern may indicate that stimulation of thyroid activity by TSH occurs during the early stages of thyroid gland histodifferentiation in *E. coqui*. Immunoreactive TSH is present in the pituitary of *E. coqui* at stage 9, before the thyroid has fully differentiated, which is consistent with pituitary stimulation of the thyroid gland during initial stages of thyroid differentiation (Jennings, 1997).

Thyroid Activity and the Formation of Adult Features

Given the prominent role of TH in regulating amphibian metamorphosis, a number of authors have suggested that peak thyroid gland activity should correlate with the appearance of metamorphic features and the length of the larval period (Iwasawa, 1966; Francois-Krassowska, 1989). In species with relatively short larval periods, such as *R. tagoi*, *B. japonicus*, and *P. fuscus*, peak thyroid activity occurs relatively early, at the time of forelimb emergence (Iwasawa, 1966; Francois-Krassowska, 1982). Species with longer development times, such as *Rh. ornatoventris*, *Rh. arboreus*, and *B. calamita*, exhibit a later peak in thyroid measures, at the onset of tail regression (Iwasawa, 1966; Francois-Krassowska, 1989). In species with very long larval periods, such as *R. catesbeiana*, peak thyroid activity occurs much later, during metamorphic climax, and it is correlated with final stages of tail regression (Iwasawa, 1966). Although quantitative changes in thyroid histology have been used frequently to gauge thyroid activity, many studies demonstrate that such measures may be a crude indicator of thyroid activity at best. For example, epithelial cell height does not correlate with circulating levels of TH in the tiger salamander, *Ambystoma tigrinum* (Norman *et al.*, 1987). In addition, the thyroid begins to accumulate iodine and synthesize thyroid hormones before it is fully differentiated in *B. japonicus* (Hanaoka *et al.*, 1973), *Hyla v. versicolor* (Lynn and Dent, 1958), and *X. laevis* (Shellabarger and Brown, 1959).

In *E. coqui*, the general pattern of a peak in thyroid activity followed by a decrease in activity prior to hatching was observed in two thyroid measures. Follicle number and follicle volume both peak during late embryogenesis (TS 13) and then decrease prior to hatching (TS 15; Figs. 2B and 2C). Embryonic peaks in these measures are not correlated with forelimb emergence, as forelimbs develop very early in *E. coqui* (TS 4) and are never enclosed by an operculum. Several other morphological transitions, however, which are comparable to metamorphic changes in frogs with the ancestral life history, are correlated with thyroid activity in *E. coqui*. One such feature is tail regression, which

begins at stage 13, coincident with peak thyroid activity (Townsend and Stewart, 1985). A second feature is remodeling of the hyobranchial skeleton (Hanken *et al.*, 1992). Before the thyroid develops, the hyobranchial skeleton of *E. coqui* resembles the larval structure of frogs with a biphasic life history. Initial remodeling of this ''larval'' hyobranchial skeleton to a ''midmetamorphic'' configuration begins at stage 9 and is complete by stage 10. A second period of remodeling occurs between stages 12 and 14, when the hyobranchial skeleton assumes a ''postmetamorphic'' morphology. Both of these transitions coincide with discrete phases of thyroid development: initial remodeling coincides with thyroid differentiation, whereas the second phase of remodeling correlates with the embryonic peaks in follicle number and follicle volume. Jaw muscle development is a third feature (Hanken *et al.*, 1997b). In embryonic *E. coqui*, the jaw-opening musculature initially (TS 7) assumes a mid-metamorphic configuration, which includes several independent muscle rudiments that correspond to larval-specific muscles of metamorphosing frogs. Between stages 12 and 15 these muscle rudiments reorient, shift origins and attachments, and fuse to form the single, complex jaw-opening muscle (m. depressor mandibulae) found in adult frogs. The morphological changes that occur after stage 12, which closely resemble metamorphic transitions in frogs that display the ancestral, biphasic life history, are correlated with embryonic peaks in thyroid activity.

Presence of colloid vacuoles starting at TS 12 may indicate release of TH to the circulation at this time (Fox, 1966; but see McNabb, 1992), coincident with the remodeling of cranial cartilage and muscle. Colloid vacuoles have been observed during early stages of metamorphosis in amphibians with the ancestral, biphasic life history (Fox, 1966; Francois-Krassowska, 1989) and in amphibians with derived life histories (e.g., *Arthroleptella villiersii*, Brink, 1939). While this may indicate that simultaneous production and release of TH during early metamorphosis is a general feature of frogs, vacuoles are not as common in metamorphic *B. angusticeps* as they are in *Arthroleptella* (Brink, 1939).

Quantitative changes in two measures of thyroid histology during embryogenesis in *E. coqui* parallel morphological changes that correspond to those that occur during metamorphosis in amphibians with the ancestral life history. Embryonic peaks in both follicle number and volume are followed closely by morphological remodeling in a number of peripheral tissues. These results support the hypothesis that precocious, embryonic development and activity of the thyroid gland is one of the mechanisms that underlies the evolution of direct development in anuran amphibians. However, the exact role of the thyroid gland, as well as other components of the thyroid axis, in the embryonic development of *E. coqui* and other direct-developing amphibians remains to be defined. Additional analyses are also needed to more fully assess the possible complementary role played by reduction or loss of TH-dependence by peripheral tissues in the evolution of direct development (Lynn and Peadon, 1955; Hughes 1966; Hughes and Reier, 1972; Elinson, 1994; Callery and Elinson, 1996).

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