General and Comparative Endocrinology 214 (2015) 62–67

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Development of neuroendocrine components of the thyroid axis in the direct-developing frog *Eleutherodactylus coqui*: Formation of the median eminence and onset of pituitary TSH production

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ARTICLE INFO

Article history:
Received 9 September 2014
Revised 21 January 2015
Accepted 24 January 2015
Available online 5 March 2015

Keywords:
Direct development
Thyroid axis
Median eminence
Thyroid-stimulating hormone
Metamorphosis

ABSTRACT

Direct-developing frogs lack, wholly or in part, a wide range of larval features found in metamorphosing species and form adult-specific features precociously, during embryogenesis. Most information on thyroid regulation of direct development relies on hormone manipulations; the ontogeny of many thyroid axis components has not been fully described. This analysis examines differentiation of the median eminence of the hypothalamus and production of thyroid-stimulating hormone (TSH) by the pituitary of the direct-developing frog *Eleutherodactylus coqui*. The median eminence is established two-thirds of the way through embryogenesis. Cells immunoreactive to human TSHβ antibodies are first detected during embryogenesis and quantitative changes in TSHβ-IR cells resemble those in metamorphosing amphibians. Formation of the median eminence of the hypothalamus and TSHβ production by the pituitary precede or coincide with morphological changes during embryogenesis that occur during metamorphosis in biphasic anurans. Thus, while the onset of neuroendocrine regulation has changed during the evolution of direct development, it is likely that these thyroid axis components still mediate the formation of adult features.

1. Introduction

Amphibian metamorphosis is a well-established model for examining the developmental role of hormones, particularly thyroid hormone (TH). Recently, there has been increased emphasis on expanding the diversity of amphibian species examined and on evaluating the role of endocrine mechanisms in mediating evolutionary changes in metamorphic life history strategies (Buchholz et al., 2011). The most familiar, and phylogenetically ancestral, life history in amphibians is biphasic; embryogenesis produces a free-living larval stage that is then extensively remodeled during a second discrete phase of development, metamorphosis. Evolutionary changes in this pattern range from shortening or lengthening the larval period to elimination of either the adult or the free-living larval stage. The latter change results in direct development, in which the adult (*i.e.*, postmetamorphic) anatomy forms precociously, during embryogenesis. Mechanistically, such diversification likely results from alterations in the thyroid axis, the primary endocrine regulator of metamorphosis (Buchholz et al., 2011; Page et al., 2009; Safi et al., 2006; Elinson, 2013). Like most endocrine axes, the thyroid axis consists of a series of central regulators that mediate the production and release of TH from the thyroid gland and peripheral regulators that mediate tissue-specific responses to circulating hormone (Buchholz et al., 2011). Alterations that affect metamorphic timing potentially occur at any level of the thyroid axis, and evolutionary changes at one level will often have effects at other levels.

Ontogenetic reduction of larval features and formation of adult anatomy in direct-developing amphibians are potentially mediated by TH, as they are in metamorphic amphibians. Although direct development is a phylogenetically widespread life-history strategy, having evolved independently numerous times in both frogs and salamanders, most studies that examine TH regulation of direct development have focused on a single species of frog, *Eleutherodactylus coqui* (Elinson, 2013). In *E. coqui*, many tadpole features are reduced or absent, the notable exception being the tail, which is prominent, at least in the embryo (Townsend and Stewart, 1985). Several other features initially assume a mid-metamorphic

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http://dx.doi.org/10.1016/j.ygcen.2015.01.029
0016-6480/

configuration before being remodeled to the adult morphology (*e.g.*, cranial cartilages and muscles; Hanken et al., 1992, 1997; Ziermann and Diogo, 2014). Limb and spinal cord development are also accelerated in this species; each forms much earlier than in metamorphic frogs (Elinson, 2013; Schlosser, 2003).

The role of TH in mediating embryonic development in *E. coqui* has been assessed primarily through hormone manipulations, which demonstrate that exogenous TH—or TH inhibitors—alter the timing or extent of morphological change (reviewed in Elinson, 2013). More recent manipulations extend beyond direct alteration of TH by instead altering hypothalamic thyroid axis components that regulate TH production and release in metamorphic species (Kulkarni et al., 2010). Hypothalamic hormone manipulations alter the timing and extent of morphological changes comparable to metamorphosis—results similar to those from TH manipulations. Together, these results suggest that a wide range of features of developing *E. coqui* remain responsive to TH, but few studies directly examine the ontogeny of specific components of the thyroid axis.

In this study, we examine the differentiation of the median eminence of the hypothalamus and ontogenetic changes in TSH production by the pituitary in *E. coqui*. Detailed descriptions of the development of thyroid axis components in *E. coqui* are available only for the thyroid gland (Jennings and Hanken, 1998) and for mRNA levels of TH receptors (Callery and Elinson, 2000). If neuroendocrine control of the thyroid axis is involved in the evolution of direct development, then development of the median eminence of the hypothalamus and onset of pituitary TSH production in *E. coqui* should occur during embryogenesis. In addition, onset of neuroendocrine regulation of thyroid activity should precede or coincide with morphological changes that resemble metamorphic changes seen in other frogs.

2. Materials and methods

2.1. Animal care

A developmental series of embryonic *E. coqui* was obtained from spontaneous matings among wild-caught adults maintained as a laboratory breeding colony at the University of Colorado Boulder (Elinson et al., 1990; Hanken et al., 1992; Moury and Hanken, 1995). After removal of the attending male, eggs were cultured in Petri dishes lined with filter paper moistened with 10% Holtfreter solution. Petri dishes were covered and placed in an incubator at 25 °C.

Animal-care procedures are approved by the University of Colorado Boulder Institutional Animal Care and Use Committee. An Animal Welfare Assurance statement is on file with the university's Office of Animal Resources.

Adult frogs 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), as part of the Long-Term Ecological Research Program in the Luquillo Experimental Forest.

2.2. Staging and samples

Embryos were staged according to Townsend and Stewart (1985), a staging table specific for *E. coqui*. Samples included embryos from multiple unrelated clutches ($n = 2$ clutches for median eminence histology, $n > 2$ clutches for TSHβ immunohistochemistry).

2.3. Median eminence histology

Embryos were fixed in 10% neutral-buffered formalin, dehydrated, and embedded in Paraplast. Serial sagittal sections (6 μm) of entire embryos were stained with a four-part connective tissue stain (Alcian blue, direct red, Celestine blue and hematoxylin; Hall, 1985). A total of 2 specimens each from stages 8 to 15 were prepared. The following features of the median eminence were examined: shape of the median eminence, presence of an ependymal layer, nerve fibers that form the internal zone, and appearance of capillaries that form the external zone between the anterior pituitary and the infundibulum.

2.4. TSHβ immunohistochemistry

Embryos were fixed in Dent fixative (1 part DMSO: 4 parts methanol; Dent et al., 1989), dehydrated in ethanol, and embedded in Paraplast. Sagittal serial sections (6 μm) were prepared and immunostained using a peroxidase–antiperoxidase technique. After pre-blocking with normal goat serum, slides were incubated overnight with rabbit anti-human beta TSH (National Hormone and Pituitary Program [NHPP], lot #AFP55741789) diluted 1:500 in serum cocktail (5% newborn calf serum, 5% DMSO, 0.1% thimerosal, 0.4% Triton X-100 in 0.1 M phosphate [K/Na]-buffered saline [Niu-Twitty salts]). Sections were rinsed with phosphate-buffered saline (PBS; pH 7.4) and treated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) diluted 1:1000 in serum cocktail. To visualize antibody staining, slides were reacted with 0.5 mg/ml diaminobenzidine (Sigma Chemical Co., St. Louis, Mo.) and 0.02% hydrogen peroxide in PBS for 1.5 h. Slides were then counterstained with Alcian blue. Two different controls were used to confirm the specificity of antibody staining. In one set of embryos, the primary antibody was replaced with non-immune rabbit serum. A second set of embryos was treated with primary antibody that had been pre-absorbed by incubation with ovine TSHβ (NHPP, lot #AFP-3748B).

2.5. Quantification of TSHβ immunohistochemistry

Measurements were made using a Leitz Dialux 20 compound microscope and a DEI-470 Optronics video camera attached to a computer equipped with NIH-image. Total pituitary area and the area of TSHβ-immunoreactive (IR) cells were measured in each section. Measurements were taken on five specimens per stage from stages 9 to 15. Pituitary and TSHβ-IR cell volume were calculated by multiplying area measurements by section thickness. Percent TSHβ-IR cell volume for each specimen was calculated by dividing TSHβ-IR cell volume by pituitary volume and multiplying by 100. Data in the text are presented as means ± one standard error.

2.6. Statistics for TSHβ immunohistochemistry

Data were analyzed by Tukey-HSD analysis of variance performed using SPSS.

3. Results

3.1. Ontogeny of the median eminence

At TS 8, the earliest embryonic stage examined, the median eminence of *E. coqui* consists of a thin epithelial layer composed primarily of cuboidal cells that separate the third ventricle from the pituitary anlage (Fig. 1A). Anteriorly, the epithelial layer is thicker, less distinct, and blends with underlying cells. The median eminence at TS 9 differs little from stage 8. By TS 10, the epithelium forms a distinct ependymal layer composed of columnar cells that form a continuous layer between the third ventricle and the underlying pituitary (Fig. 1B). Directly under the ependymal layer,

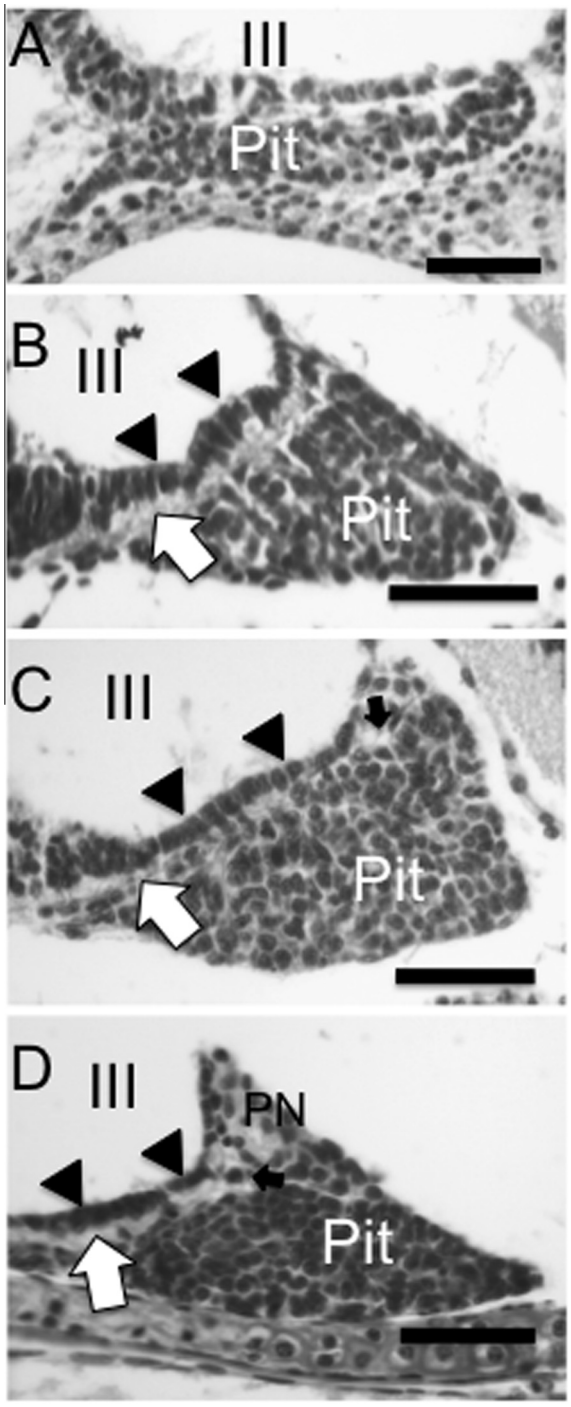

Fig. 1. Sagittal sections (6 μm) through the median eminence-pituitary of *E. coqui* embryos stained with Alcian blue, direct red, Celestine blue and hematoxylin (Hall, 1985). Anterior is to the left. (A) Townsend and Stewart (1985) stage 8, (B) stage 10, (C) stage 12, (D) stage 14. Arrowheads: columnar epithelia forming ependymal cell layer. Large open arrows: internal zone formed of nerve fibers and lacking nuclei. Small arrows: capillaries characteristic of the external zone. *Abbreviations:* III, third ventricle; Pit, pituitary; PN, pars nervosa. Scale bars: 10 μm.

nerve fibers of the internal zone have begun to form between the epithelium and the pituitary. Throughout the remainder of embryogenesis (TS 11–15) the ependymal layer and underlying nerve fibers are present. Small blood vessels forming the external zone, are present in several sections between the internal zone and the pituitary (Fig. 1C and D). At stage 14, the posterior lobe of the pituitary is distinct from the anterior lobe (Fig. 1D).

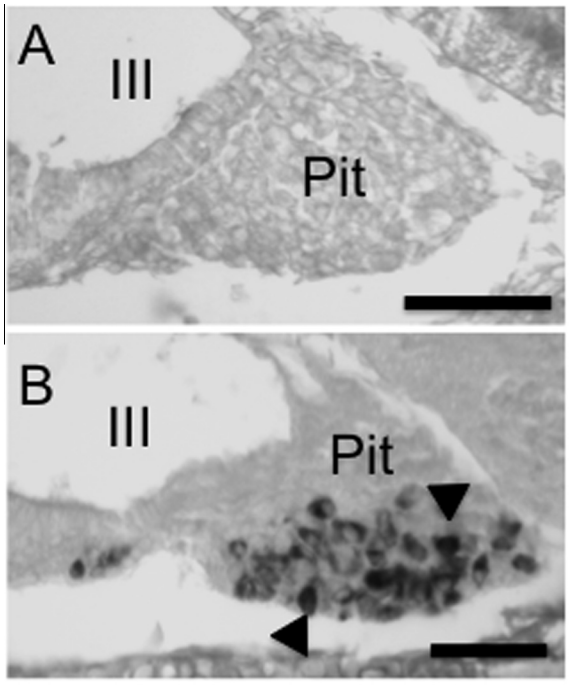

Fig. 2. Sagittal sections (6 μm) through the pituitary of *E. coqui* embryos at TS stage 10. Anterior is to the left. (A) Control section stained with human TSHβ antibody preabsorbed with ovine TSH. No specific staining is apparent. (B) Section immunostained with human TSHβ. TSHβ-positive cells (arrowheads) appear dark. *Abbreviations:* III, third ventricle; Pit, pituitary. Scale bars: 10 μm.

3.2. Ontogeny of pituitary TSHβ production

3.2.1. Immunohistochemical controls

No positive staining was seen when sections were incubated with normal rabbit serum in place of the primary antibody or when the primary antibody was preabsorbed with ovine TSHβ (Fig. 2A).

3.2.2. Localization and ontogeny of TSHβ immunoreactive cells in the pituitary

Cells immunoreactive to rabbit anti-human TSHβ antibodies were observed in the mid-ventral portion of the pars distalis of embryonic *E. coqui* (Fig. 2B). Immunoreactivity was first observed at TS 9, when all but one specimens stained positively for TSHβ. In contrast, no specimen at TS 8 stained with this antibody. Throughout the remainder of embryogenesis (TS 10–15), positive immunoreactivity was found in the ventral pars distalis of all specimens.

3.2.3. Quantitative analysis of pituitary development and TSHβ-IR cell volume

Quantitative measurements of pituitary volume, TSHβ-IR volume, and percent TSHβ volume are summarized in Fig. 3. Pituitary volume increases significantly between stage 9 and stages 13–15 but does not differ among stages 10–15 ($F = 3.085$, $p < 0.05$; Fig. 3A). The volume of TSHβ-IR cells increases between stages 9/10 and stage 13, but does not differ among stages 11–12 or 14–15 ($F = 6.335$, $p < 0.05$; Fig. 3B). Changes in percent TSHβ-IR cell volume exhibit the same pattern as TSHβ-IR cell volume ($F = 6.683$, $p < 0.05$; Fig. 3C).

4. Discussion

Onset of neuroendocrine regulation of thyroid activity represents a fundamental control of the initiation and rate of amphibian

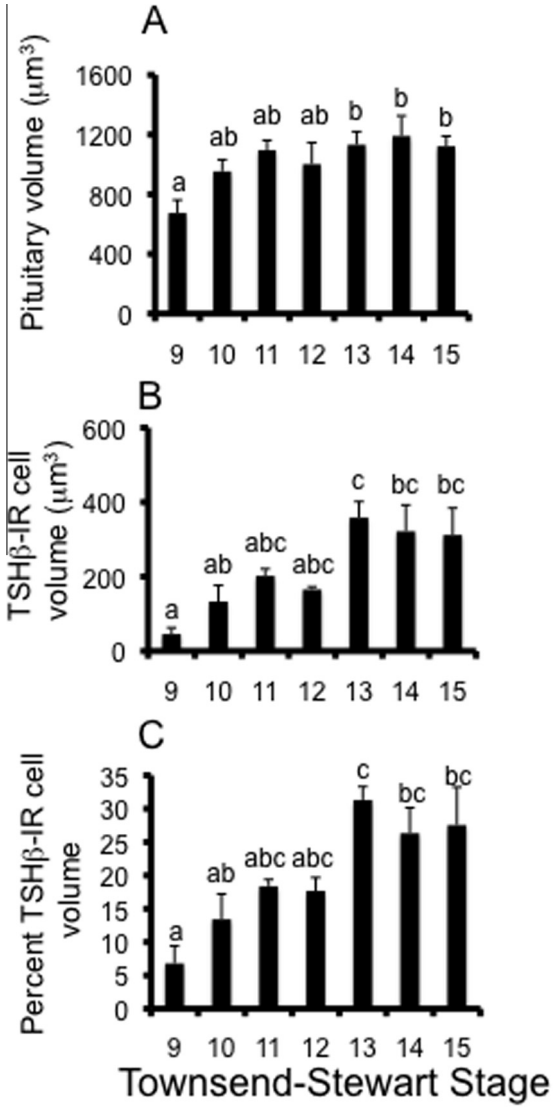

Fig. 3. Histograms of pituitary and TSHβ measurements. Each bar represents the mean of five specimens ±1 SEM. Groups sharing superscripts are not significantly different (Tukey-HSD, $p > 0.05$). (A) Pituitary volume, (B) volume of TSHβ-immunoreactive cells, (C) percent TSHβ volume.

metamorphosis (Denver, 2013). In larvae, such control is mediated through the hypothalamus and pituitary. The hypothalamus produces a number of neurohormones that, once transported to the pituitary via the internal and external zones of the median eminence, regulate pituitary function. In response to hypothalamic stimulation, the pituitary produces several tropic hormones, including TSH, which regulates TH release and, hence, metamorphosis. The current study is the first to report the ontogeny of median eminence formation in any species of *Eleutherodactylus*, and the first to document the ontogeny and activity of pituitary TSHβ production in direct-developing amphibians.

4.1. Hypothalamic regulation of direct development

Hypothalamic signals reach the pituitary through the median eminence, which is comprised of nerve fibers (internal zone) that connect hypothalamic nuclei to the posterior pituitary, and blood vessels of the hypophyseal portal system (external zone). In metamorphic amphibians, maturation of transport systems connecting the median eminence to the pituitary are associated with increased pituitary TSH levels and circulating thyroid hormone levels (Denver, 2013). In *E. coqui*, the nerve fibers of the internal zone of the median eminence are present at TS 10, and capillaries of the external zone are identifiable shortly afterwards (Fig. 1B–D). Formation of the median eminence coincides with initial stages of thyroid differentiation but precedes the formation of organized colloid-filled follicles (Jennings and Hanken, 1998). In metamorphosing frogs, development of the median eminence is dependent on TH; the median eminence is not fully differentiated until after formation of the larval thyroid (reviewed in Denver, 2013). While median eminence development in *E. coqui* occurs prior to formation of colloid-filled follicles, thyroid hormones may still play a role in hypothalamic development. Neural tissues in metamorphic frogs respond to relatively low levels of TH (Denver, 2013) and TH synthesis in the developing thyroid of *E. coqui* may still be sufficient to mediate hypothalamic development. Maternal provisioning is also a potential source of TH prior to the formation of the embryonic thyroid. Levels of TH during *E. coqui* development have yet to be reported.

Differentiation of the median eminence in *E. coqui* precedes morphological changes during embryogenesis, such as tail regression and cranial cartilage and muscle remodeling, that occur during metamorphosis in amphibians with the ancestral, biphasic life history (Fig. 4; Elinson, 2013; Hanken et al., 1992, 1997; Ziermann and Diogo, 2014). The only published account of median eminence ontogeny in a frog with a derived life history is for *Arthroleptella lightfooti*, which passes through a non-feeding, terrestrial larval stage (Morgan et al., 1989). Formation of the median eminence in this species occurs post-hatching, but before the onset of exogenous feeding, and is correlated with the appearance of several post-metamorphic features such as pronephros degeneration, skin metamorphosis, and tail resorption.

In larval anurans, hypothalamic regulation of pituitary thyroid-stimulating hormone (TSH) is mediated by corticotropin-releasing factor (CRF), which also regulates adrenocorticotropic hormone production and release (Denver, 2013). A similar control system has been documented for *E. coqui*; treatment with CRF accelerates morphological changes such as tail resorption, whereas treatment with a CRF receptor antagonist (astressin) delays it (Kulkarni et al., 2010). Our data are consistent with these results and demonstrate that the hypothalamic transport system is present in *E. coqui* during the stages when CRF manipulations are capable of altering development. However, there are no published data regarding endogenous production or release of CRF from the hypothalamus of *E. coqui*.

4.2. Pituitary regulation of direct development

4.2.1. Ontogenetic comparisons

In *E. coqui*, TSHβ-IR cells are located in the mid-ventral portion of the pituitary. A similar distribution of TSHβ-IR cells is seen by using antibodies to hTSHβ in adults of all three extant amphibian orders and in larvae of salamanders and frogs (Yamashita et al., 1991; Oota and Saga, 1991; Kikuyama et al., 1993). In bullfrogs, antibodies to bullfrog TSHβ recognize the same cells as the hTSHβ antibody (Okada et al., 2004). In *E. coqui*, TSHβ-IR cells first appear at embryonic stage TS 9, immediately preceding formation of the thyroid gland at TS 10 (Jennings and Hanken, 1998). In metamorphosing frogs, TSHβ-IR cells first appear in premetamorphic larvae shortly after hatching and coincident with formation of the larval thyroid gland (Kikuyama et al., 1993; Denver, 2013). Overall, the relationship among onset of TSHβ-IR protein production by the pituitary, formation of the median eminence, and thyroid differentiation is similar between *E. coqui* and metamorphic frogs: formation and activity of all these thyroid axis components occur prior to formation of many adult features (Fig. 4).

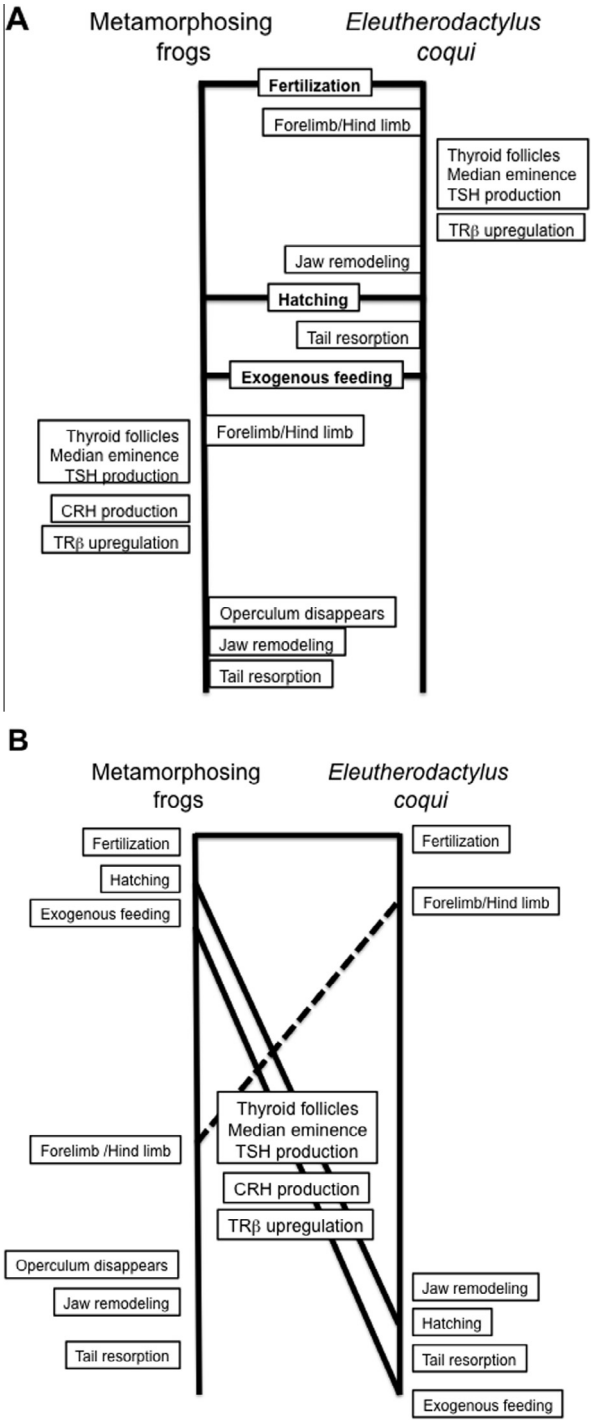

Fig. 4. Comparison of thyroid-axis formation in metamorphic frogs (left) and direct-developing *E. coqui* (right). (A) Different life-history strategies are standardized with respect to the timing of fertilization, hatching and onset of exogenous feeding (central column, bold font). The relative timing of tissue remodeling and the formation of several adult features are shown on either side of the vertical bars. Features in the same box form concurrently or their relative timing varies among species. (B) Different life-history strategies are standardized with respect to the formation of thyroid axis components (central column). Features that are delayed in *E. coqui* relative to the formation of thyroid components—hatching and exogenous feeding—are connected with solid lines; features that are accelerated in *E. coqui*—fore- and hind-limb formation—are connected by dashed lines. Morphological data for metamorphosing frogs are from Nieuwkoop and Faber (1956) and Gosner (1960). Thyroid axis components are from Kikuyama et al. (1993) and Denver (2013). Morphological data for *E. coqui* are from Townsend and Stewart (1985) and Elinson (2013). Thyroid axis data for *E. coqui* are from Callery and Elinson (2000), Jennings and Hanken (1998), and this study.

4.2.2. Quantitative comparisons

Although the ontogeny of TSHβ-IR protein production in larvae has been documented for a number of amphibian species, there are few quantitative analyses of changes in TSHβ-IR cells throughout development. In *E. coqui*, the volume of TSHβ-IR cells increases from TS 9 to TS 13 and remains at this level until hatching (TS 15; Fig. 3B). Such quantitative changes in embryonic *E. coqui* partly parallel changes seen during metamorphosis in other species. Similar to *E. coqui*, in metamorphosing frogs the number and area of TSHβ-IR cells increase until the early stages of metamorphic climax, although TSHβ-IR decreases prior to the completion of metamorphosis (Yamashita et al., 1991; Garcia-Navarro et al., 1988; Kurabuchi et al., 1987). Quantitative changes of TSHβ mRNA levels throughout metamorphosis parallel those observed in TSH cells using immunohistochemical methods; TSHβ mRNA levels rise during early climax and then decline before metamorphosis is complete (Buckbinder and Brown, 1993; Okada et al., 2009). Finally, direct measures of TSH proteins in the pituitary or plasma of metamorphic amphibians increase through late climax stages before declining (Okada et al., 2009; Korte et al., 2011).

Initial increase in pituitary TSH in metamorphic frogs coincides with formation of the median eminence, which suggests that hypothalamic hormones stimulate TSH production during early metamorphic stages (Manzon and Denver, 2004). In *E. coqui*, the volume of TSHβ-IR cells increases dramatically between stages 12 and 13, possibly indicating increased stimulation of TSH cells by hypothalamic hormones. Differentiation of the median eminence of *E. coqui* at TS 10, prior to the rapid increase in TSHβ-IR cell volume, is similarly consistent with hypothalamic stimulation of pituitary activity during these stages. In all these frogs, several measures of thyroid histology, including follicle number, follicle volume, colloid volume, and epithelial cell height peak before the decline in measures of TSH volume (Jennings and Hanken, 1998). Given that TSH regulates metamorphosis through its effects on thyroid activity, the pattern of histological changes observed in the thyroid gland is consistent with TSH stimulation of the thyroid occurring shortly after TSHβ-IR cells first appear. In addition, TSHβ-IR expression in *E. coqui* is elevated during stages when CRF manipulations alter the onset and rate of morphological remodeling that is comparable to metamorphosis (Kulkarni et al., 2010).

Decline in TSHβ-IR cells during the later stages of metamorphosis has been interpreted as a period of TSH release and is correlated with high levels of circulating TH (Garcia-Navarro et al., 1988). However, declines in TSHβ-IR cells during late metamorphosis are also consistent with negative feedback of TH on pituitary production of TSH. In metamorphosing frogs, negative feedback of TH on TSH is established during premetamorphosis, and the strength of feedback effects is regulated throughout the remainder of metamorphosis (Manzon and Denver, 2004; Sternberg et al., 2011). Consequently, the decline in TSHβ-IR cell measurements during late stages of development in metamorphosing amphibians may result from negative feedback on TSH production and not just from increased TSH release.

4.3. Conclusions

Differentiation of the median eminence and onset of pituitary TSH production in the direct-developing frog *E. coqui* occur during the late embryonic period. In contrast, these features do not form in metamorphosing frogs until after hatching, during the larval period (Fig. 4A). Embryonic formation of neuroendocrine components precedes or is coincident with many morphological changes that resemble metamorphosis in biphasic anurans. Despite marked differences in morphological development relative to hatching,

integration among neuroendocrine components also appears to be conserved between *E. coqui* and metamorphic frogs, as formation and activity of components of the hypothalamic–pituitary–thyroid axis occur within a narrow range of developmental stages and in a similar sequence (Fig. 4B). These data support the hypothesis that central components of the thyroid axis in *E. coqui* function the same way that they do in metamorphic amphibians (Jennings and Hanken, 1998; Kulkarni et al., 2010; Elinson, 2013). However, feedback interactions among thyroid axis components documented in metamorphic frogs have not been examined in *E. coqui*, and a notable difference between *E. coqui* and metamorphic frogs is the lack of a significant decline in TSHβ-IR during the late stages of embryogenesis. This may indicate that TSHβ regulation in *E. coqui* differs from other frogs with respect to the onset of negative feedback interactions or responsiveness to hypothalamic signals. Additionally, thyroid axis components remain active in *E. coqui* after hatching and regulate aspects of post-hatching development (Callery and Elinson, 2000; Singamsetty and Elinson, 2010). While thyroid hormone control of late stages of *E. coqui* development appears conserved among *E. coqui* and metamorphic frogs, the role of TH in early development is unclear. Mechanisms underlying development of several features (*e.g.*, eyes, limbs) may differ markedly between metamorphic and direct developing frogs (Fig. 4A and B; Elinson, 2013).

Acknowledgments

Financial support was provided by the U.S. National Science Foundation: IBN93-21572 to D.H.J.; DCB90-I9624 and IBN94-19407 to J.H.B. Evans was supported by the Undergraduate Research and Creative Activities program at SIUE. Anti-human TSHβ antibody (lot #AFP55741789) and ovine TSHβ peptide (lot #AFP-3748B) were obtained from the National Hormone and Peptide Program (NHPP), NIDDK, and Dr. Parlow of the Harbor-UCLA Medical Center.

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