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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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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 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.

106 Animal-care procedures are approved by the University of Colorado
107 Boulder Institutional Animal Care and Use Committee. An Animal Welfare
108 Assurance statement is on file with the university's Office of Animal Resources.
109 Adult frogs were collected with the permission of the Puerto Rico Department of
110 Natural Resources (permits DRN-91–45, DRN-92–19, DRN-93–26, and DRNA-
111 95–26), as part of the Long-Term Ecological Research Program in the Luquillo
112 Experimental Forest.
113
114 **2.2 Staging and samples**
115 Embryos were staged according to Townsend and Stewart (TS; 1985), a staging
116 table specific for *E. coqui.* Samples included embryos from multiple unrelated
117 clutches (n = 2 clutches for median eminence histology, n > 2 clutches for TSHβ
118 immunohistochemistry).
119
120 **2.3 Median eminence histology**
121 Embryos were fixed in 10% neutral-buffered formalin, dehydrated, and
122 embedded in Paraplast. Serial sagittal sections (6 μm) of entire embryos were
123 stained with a four-part connective tissue stain (Alcian blue, direct red, celestine
124 blue and hematoxylin; Hall, 1985). A total of 2 specimens each from stages 8 to
125 15 were prepared. The following features of the median eminence were
126 examined: shape of the median eminence, presence of an ependymal layer,
127 nerve fibers that form the internal zone, and appearance of capillaries that form
128 the external zone between the anterior pituitary and the infundibulum.

129

130 **2.4 TSHβ immunohistochemistry**

131 Embryos were fixed in Dent fixative (1 part DMSO: 4 parts methanol; Dent *et al.,*
132 1989), dehydrated in ethanol, and embedded in Paraplast. Sagittal serial
133 sections (6 μm) were prepared and immunostained using a peroxidase-
134 antiperoxidase technique. After pre-blocking with normal goat serum, slides were
135 incubated overnight with rabbit anti-human beta TSH (National Hormone and
136 Pituitary Program [NHPP], lot #AFP55741789) diluted 1:500 in serum cocktail
137 (5% newborn calf serum, 5% DMSO, 0.1% thimerosal, 0.4% Triton X-100 in 0.1
138 M phosphate [K/Na]-buffered saline [Niu-Twitty salts]). Sections were rinsed with
139 phosphate-buffered saline (PBS; pH 7.4) and treated for 2 hr with horseradish
140 peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) diluted 1:1000 in serum
141 cocktail. To visualize antibody staining, slides were reacted with 0.5 mg/ml
142 diaminobenzidine (Sigma Chemical Co., St. Louis, Mo.) and 0.02% hydrogen
143 peroxide in PBS for 1.5 hr. Slides were then counterstained with Alcian blue. Two
144 different controls were used to confirm the specificity of antibody staining. In one
145 set of embryos, the primary antibody was replaced with non-immune rabbit
146 serum. A second set of embryos was treated with primary antibody that had been
147 pre-absorbed by incubation with ovine TSHβ (NHPP, lot #AFP-3748B).

148

149 **2.5 Quantification of TSHβ immunohistochemistry**

150 Measurements were made using a Leitz Dialux 20 compound microscope and a
151 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, 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,D). At stage 14, the posterior lobe of the pituitary is distinct from the anterior lobe (Fig. 1D).

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 Figure 3. Pituitary volume increases

198 significantly between stage 9 and stages 13–15 but does not differ among stages
199 10–15 ($F = 3.085$, $P < 0.05$; Fig. 3A). The volume of TSHβ-IR cells increases
200 between stages 9/10 and stage 13, but does not differ among stages 11–12 or
201 14–15 ($F = 6.335$, $p < 0.05$; Fig. 3B). Changes in percent TSHβ-IR cell volume
202 exhibit the same pattern as TSHβ-IR cell volume ($F = 6.683$, $p < 0.05$; Fig. 3C).
203
204 **4. Discussion**
205 Onset of neuroendocrine regulation of thyroid activity represents a fundamental
206 control of the initiation and rate of amphibian metamorphosis (Denver, 2013). In
207 larvae, such control is mediated through the hypothalamus and pituitary. The
208 hypothalamus produces a number of neurohormones that, once transported to
209 the pituitary via the internal and external zones of the median eminence, regulate
210 pituitary function. In response to hypothalamic stimulation, the pituitary produces
211 several tropic hormones, including TSH, which regulates TH release and, hence,
212 metamorphosis. The current study is the first to report the ontogeny of median
213 eminence formation in any species of *Eleutherodactylus,* and the first to
214 document the ontogeny and activity of pituitary TSHβ production in direct-
215 developing amphibians.
216
217 **4.1 Hypothalamic regulation of direct development**
218 Hypothalamic signals reach the pituitary through the median eminence, which is
219 comprised of nerve fibers (internal zone) that connect hypothalamic nuclei to the
220 posterior pituitary, and blood vessels of the hypophyseal portal system (external

221 zone). In metamorphic amphibians, maturation of transport systems connecting
222 the median eminence to the pituitary are associated with increased pituitary TSH
223 levels and circulating thyroid hormone levels (Denver, 2013). In *E. coqui,* the
224 nerve fibers of the internal zone of the median eminence are present at TS 10,
225 and capillaries of the external zone are identifiable shortly afterwards (Fig. 1B–D).
226 Formation of the median eminence coincides with initial stages of thyroid
227 differentiation but precedes the formation of organized colloid-filled follicles
228 (Jennings and Hanken, 1998). In metamorphosing frogs, development of the
229 median eminence is dependent on TH; the median eminence is not fully
230 differentiated until after formation of the larval thyroid (reviewed in Denver, 2013).
231 While median eminence development in *E. coqui* occurs prior to formation of
232 colloid-filled follicles, thyroid hormones may still play a role in hypothalamic
233 development. Neural tissues in metamorphic frogs respond to relatively low
234 levels of TH (Denver, 2013) and TH synthesis in the developing thyroid of *E.*
235 *coqui* may still be sufficient to mediate hypothalamic development. Maternal
236 provisioning is also a potential source of TH prior to the formation of the
237 embryonic thyroid. Levels of TH during *E. coqui* development have yet to be
238 reported.
239 Differentiation of the median eminence in *E. coqui* precedes morphological
240 changes during embryogenesis, such as tail regression and cranial cartilage and
241 muscle remodeling, that occur during metamorphosis in amphibians with the
242 ancestral, biphasic life history (Fig. 4; Elinson, 2013; Hanken *et al*., 1992, 1997;
243 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

267 frogs (Yamashita *et al.,* 1991; Oota and Saga, 1991; Kikuyama *et al.,* 1993). In
268 bullfrogs, antibodies to bullfrog TSHβ recognize the same cells as the hTSHβ
269 antibody (Okada *et al*., 2004). In *E. coqui,* TSHβ-IR cells first appear at
270 embryonic stage TS 9, immediately preceding formation of the thyroid gland at
271 TS 10 (Jennings and Hanken, 1998). In metamorphosing frogs, TSHβ-IR cells
272 first appear in premetamorphic larvae shortly after hatching and coincident with
273 formation of the larval thyroid gland (Kikuyama *et al.,* 1993; Denver, 2013).
274 Overall, the relationship among onset of TSHβ-IR protein production by the
275 pituitary, formation of the median eminence, and thyroid differentiation is similar
276 between *E. coqui* and metamorphic frogs: formation and activity of all these
277 thyroid axis components occur prior to formation of many adult features (Fig. 4).
278
279 **4.2.2 Quantitative comparisons**
280 Although the ontogeny of TSHβ-IR protein production in larvae has been
281 documented for a number of amphibian species, there are few quantitative
282 analyses of changes in TSHβ-IR cells throughout development. In *E. coqui,* the
283 volume of TSHβ-IR cells increases from TS 9 to TS 13 and remains at this level
284 until hatching (TS 15; Fig. 3B). Such quantitative changes in embryonic *E. coqui*
285 partly parallel changes seen during metamorphosis in other species. Similar to *E.*
286 *coqui*, in metamorphosing frogs the number and area of TSHβ-IR cells increase
287 until the early stages of metamorphic climax, although TSHβ-IR decreases prior
288 to the completion of metamorphosis (Yamashita *et al.,* 1991; Garcia-Navarro *et*
289 *al.,* 1988; Kurabuchi *et al.,* 1987). Quantitative changes of TSHβ mRNA levels

290 throughout metamorphosis parallel those observed in TSH cells using
291 immunohistochemical methods; TSHβ mRNA levels rise during early climax and
292 then decline before metamorphosis is complete (Buckbinder and Brown, 1993;
293 Okada *et al.*, 2009). Finally, direct measures of TSH proteins in the pituitary or
294 plasma of metamorphic amphibians increase through late climax stages before
295 declining (Okada *et al.*, 2009; Korte *et al.*, 2011).
296 Initial increase in pituitary TSH in metamorphic frogs coincides with
297 formation of the median eminence, which suggests that hypothalamic hormones
298 stimulate TSH production during early metamorphic stages (Manzon and Denver,
299 2004). In *E. coqui*, the volume of TSHβ-IR cells increases dramatically between
300 stages 12 and 13, possibly indicating increased stimulation of TSH cells by
301 hypothalamic hormones. Differentiation of the median eminence of *E. coqui* at TS
302 10, prior to the rapid increase in TSHβ-IR cell volume, is similarly consistent with
303 hypothalamic stimulation of pituitary activity during these stages. In all these
304 frogs, several measures of thyroid histology, including follicle number, follicle
305 volume, colloid volume, and epithelial cell height peak before the decline in
306 measures of TSH volume (Jennings and Hanken, 1998). Given that TSH
307 regulates metamorphosis through its effects on thyroid activity, the pattern of
308 histological changes observed in the thyroid gland is consistent with TSH
309 stimulation of the thyroid occurring shortly after TSHβ-IR cells first appear. In
310 addition, TSHβ-IR expression in *E. coqui* is elevated during stages when CRF
311 manipulations alter the onset and rate of morphological remodeling that is
312 comparable to metamorphosis (Kulkarni *et al.,* 2010).

313 Decline in TSHβ-IR cells during the later stages of metamorphosis has
314 been interpreted as a period of TSH release and is correlated with high levels of
315 circulating TH (Garcia-Navarro *et al.,* 1988). However, declines in TSHβ-IR cells
316 during late metamorphosis are also consistent with negative feedback of TH on
317 pituitary production of TSH. In metamorphosing frogs, negative feedback of TH
318 on TSH is established during premetamorphosis, and the strength of feedback
319 effects is regulated throughout the remainder of metamorphosis (Manzon and
320 Denver, 2004; Sternberg *et al*., 2011). Consequently, the decline in TSHβ-IR cell
321 measurements during late stages of development in metamorphosing
322 amphibians may result from negative feedback on TSH production and not just
323 from increased TSH release.
324
325 **4.3 Conclusions**
326 Differentiation of the median eminence and onset of pituitary TSH production in
327 the direct-developing frog *E. coqui* occur during the late embryonic period. In
328 contrast, these features do not form in metamorphosing frogs until after hatching,
329 during the larval period (Fig. 4A). Embryonic formation of neuroendocrine
330 components precedes or is coincident with many morphological changes that
331 resemble metamorphosis in biphasic anurans. Despite marked differences in
332 morphological development relative to hatching, integration among
333 neuroendocrine components also appears to be conserved between *E. coqui* and
334 metamorphic frogs, as formation and activity of components of the hypothalamic-
335 pituitary-thyroid axis occur within a narrow range of developmental stages and in

336 a similar sequence (Fig. 4B). These data support the hypothesis that central
337 components of the thyroid axis in *E. coqui* function the same way that they do in
338 metamorphic amphibians (Jennings and Hanken, 1998; Kulkarni *et al.*, 2010;
339 Elinson, 2013). However, feedback interactions among thyroid axis components
340 documented in metamorphic frogs have not been examined in *E. coqui,* and a
341 notable difference between *E. coqui* and metamorphic frogs is the lack of a
342 significant decline in TSHβ-IR during the late stages of embryogenesis. This may
343 indicate that TSHβ regulation in *E. coqui* differs from other frogs with respect to
344 the onset of negative feedback interactions or responsiveness to hypothalamic
345 signals. Additionally, thyroid axis components remain active in *E. coqui* after
346 hatching and regulate aspects of post-hatching development (Callery and Elinson,
347 2000; Singamsetty and Elinson, 2010). While thyroid hormone control of late
348 stages of *E. coqui* development appears conserved among *E. coqui* and
349 metamorphic frogs, the role of TH in early development is unclear. Mechanisms
350 underlying development of several features (e.g., eyes, limbs) may differ
351 markedly between metamorphic and direct developing frogs (Fig. 4A,B; Elinson,
352 2013).
353

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FIGURE LEGENDS

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 (TS; 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.

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.

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 THSβ volume.

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

511 exogenous feeding (central column, bold font). The relative timing of tissue
512 remodeling and the formation of several adult features are shown on either side
513 of the vertical bars. Features in the same box form concurrently or their relative
514 timing varies among species. (B) Different life-history strategies are
515 standardized with respect to the formation of thyroid axis components (central
516 column). Features that are delayed in *E. coqui* relative to the formation of thyroid
517 components—hatching and exogenous feeding—are connected with solid lines;
518 features that are accelerated in *E. coqui*—fore- and hind limb formation—are
519 connected by dashed lines. Morphological data for metamorphosing frogs are
520 from Niewkoop and Faber (1956) and Gosner (1960). Thyroid axis components
521 are from Kikuyama *et al*. (1993) and Denver (2013). Morphological data for *E.*
522 *coqui* are from Townsend and Stewart (1985) and Elinson (2013). Thyroid axis
523 data for *E. coqui* are from Callery and Elinson (2000), Jennings and Hanken
524 (1998), and this study.