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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.

David H. Jennings

*Southern Illinois University Edwardsville*, [dajenni@siue.edu](mailto:dajenni@siue.edu)

Bryce Evans

*Southern Illinois University Edwardsville*

James Hanken

*Harvard University*

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**Cover Page Footnote**

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

4  
5 David H. Jennings<sup>a,\*</sup>, Bryce Evans<sup>b</sup>, and James Hanken<sup>a,1</sup>

6  
7 <sup>a</sup> Department of Environmental, Population and Organismic Biology, University of  
8 Colorado, Boulder CO 80309

9  
10 <sup>b</sup> Department of Biological Sciences, Southern Illinois University Edwardsville,  
11 Edwardsville IL 62026

12

13 \* Corresponding author. Present address: Department of Biological Sciences,  
14 Southern Illinois University Edwardsville, Edwardsville IL 62026

15 email: [dajenni@siue.edu](mailto:dajenni@siue.edu)

16

17 <sup>1</sup> Present address: Museum of Comparative Zoology, Harvard University, Cambridge,  
18 MA 02138

19

## ABSTRACT

20

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

37

38

39 **1. Introduction**

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

61           Ontogenetic reduction of larval features and formation of adult anatomy in  
62 direct-developing amphibians are potentially mediated by TH, as they are in  
63 metamorphic amphibians. Although direct development is a phylogenetically  
64 widespread life-history strategy, having evolved independently numerous times in  
65 both frogs and salamanders, most studies that examine TH regulation of direct  
66 development have focused on a single species of frog, *Eleutherodactylus coqui*  
67 (Elinson, 2013). In *E.coqui*, many tadpole features are reduced or absent, the  
68 notable exception being the tail, which is prominent, at least in the embryo  
69 (Townsend and Stewart, 1985). Several other features initially assume a mid-  
70 metamorphic configuration before being remodeled to the adult morphology (*e.g.*,  
71 cranial cartilages and muscles; Hanken *et al.*, 1992, 1997; Ziermann and Diogo,  
72 2014). Limb and spinal cord development are also accelerated in this species;  
73 each forms much earlier than in metamorphic frogs (Elinson, 2013; Schlosser,  
74 2003).

75           The role of TH in mediating embryonic development in *E. coqui* has been  
76 assessed primarily through hormone manipulations, which demonstrate that  
77 exogenous TH—or TH inhibitors—alter the timing or extent of morphological  
78 change (reviewed in Elinson, 2013). More recent manipulations extend beyond  
79 direct alteration of TH by instead altering hypothalamic thyroid axis components  
80 that regulate TH production and release in metamorphic species (Kulkarni *et al.*,  
81 2010). Hypothalamic hormone manipulations alter the timing and extent of  
82 morphological changes comparable to metamorphosis—results similar to those  
83 from TH manipulations. Together, these results suggest that a wide range of

84 features of developing *E. coqui* remain responsive to TH, but few studies directly  
85 examine the ontogeny of specific components of the thyroid axis.

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

97

## 98 **2. Materials and methods**

### 99 **2.1 Animal care**

100 A developmental series of embryonic *E. coqui* was obtained from spontaneous  
101 matings among wild-caught adults maintained as a laboratory breeding colony at  
102 the University of Colorado Boulder (Elinson *et al.*, 1990; Hanken *et al.*, 1992;  
103 Moury and Hanken, 1995). After removal of the attending male, eggs were  
104 cultured in Petri dishes lined with filter paper moistened with 10% Holtfreter  
105 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 TSH $\beta$   
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  $\mu$ m) 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 TSH $\beta$ 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  $\mu$ m) 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 TSH $\beta$  (NHPP, lot #AFP-3748B).

148

#### 149 **2.5 Quantification of TSH $\beta$ 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-

152 image. Total pituitary area and the area of TSH $\beta$ -immunoreactive (IR) cells were  
153 measured in each section. Measurements were taken on five specimens per  
154 stage from stages 9 to 15. Pituitary and TSH $\beta$ -IR cell volume were calculated by  
155 multiplying area measurements by section thickness. Percent TSH $\beta$ -IR cell  
156 volume for each specimen was calculated by dividing TSH $\beta$ -IR cell volume by  
157 pituitary volume and multiplying by 100. Data in the text are presented as means  
158 +/- one standard error.

159

## 160 **2.6 Statistics for TSH $\beta$ immunohistochemistry**

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

162

## 163 **3. Results**

### 164 **3.1 Ontogeny of the median eminence**

165 At TS 8, the earliest embryonic stage examined, the median eminence of *E.*  
166 *coqui* consists of a thin epithelial layer composed primarily of cuboidal cells that  
167 separate the third ventricle from the pituitary anlage (Fig. 1A). Anteriorly, the  
168 epithelial layer is thicker, less distinct, and blends with underlying cells. The  
169 median eminence at TS 9 differs little from stage 8. By TS 10, the epithelium  
170 forms a distinct ependymal layer composed of columnar cells that form a  
171 continuous layer between the third ventricle and the underlying pituitary (Fig. 1B).  
172 Directly under the ependymal layer, nerve fibers of the internal zone have begun  
173 to form between the epithelium and the pituitary. Throughout the remainder of  
174 embryogenesis (TS 11–15) the ependymal layer and underlying nerve fibers are

175 present. Small blood vessels forming the external zone, are present in several  
176 sections between the internal zone and the pituitary (Fig. 1C,D). At stage 14, the  
177 posterior lobe of the pituitary is distinct from the anterior lobe (Fig. 1D).

178

## 179 **3.2 Ontogeny of pituitary TSH $\beta$ production**

### 180 **3.2.1 Immunohistochemical controls**

181 No positive staining was seen when sections were incubated with normal rabbit  
182 serum in place of the primary antibody or when the primary antibody was  
183 preabsorbed with ovine TSH $\beta$  (Fig 2A).

184

### 185 **3.2.2 Localization and ontogeny of TSH $\beta$ immunoreactive cells in the** 186 **pituitary**

187 Cells immunoreactive to rabbit anti-human TSH $\beta$  antibodies were observed in the  
188 mid-ventral portion of the pars distalis of embryonic *E. coqui* (Fig. 2B).

189 Immunoreactivity was first observed at TS 9, when all but one specimens stained  
190 positively for TSH $\beta$ . In contrast, no specimen at TS 8 stained with this antibody.

191 Throughout the remainder of embryogenesis (TS 10–15), positive

192 immunoreactivity was found in the ventral pars distalis of all specimens.

193

### 194 **3.2.3 Quantitative analysis of pituitary development and TSH $\beta$ -IR cell** 195 **volume**

196 Quantitative measurements of pituitary volume, TSH $\beta$ -IR volume, and percent

197 TSH $\beta$  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 TSH $\beta$ -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 TSH $\beta$ -IR cell volume  
202 exhibit the same pattern as TSH $\beta$ -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 TSH $\beta$  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

244 ontogeny in a frog with a derived life history is for *Arthroleptella lightfooti*, which  
245 passes through a non-feeding, terrestrial larval stage (Morgan *et al.*, 1989).  
246 Formation of the median eminence in this species occurs post-hatching, but  
247 before the onset of exogenous feeding, and is correlated with the appearance of  
248 several post-metamorphic features such as pronephros degeneration, skin  
249 metamorphosis, and tail resorption.

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

261

## 262 **4.2 Pituitary regulation of direct development**

### 263 **4.2.1 Ontogenetic comparisons**

264 In *E. coqui*, TSH $\beta$ -IR cells are located in the mid-ventral portion of the pituitary. A  
265 similar distribution of TSH $\beta$ -IR cells is seen by using antibodies to hTSH $\beta$  in  
266 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 TSH $\beta$  recognize the same cells as the hTSH $\beta$   
269 antibody (Okada *et al.*, 2004). In *E. coqui*, TSH $\beta$ -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, TSH $\beta$ -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 TSH $\beta$ -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 TSH $\beta$ -IR protein production in larvae has been  
281 documented for a number of amphibian species, there are few quantitative  
282 analyses of changes in TSH $\beta$ -IR cells throughout development. In *E. coqui*, the  
283 volume of TSH $\beta$ -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 TSH $\beta$ -IR cells increase  
287 until the early stages of metamorphic climax, although TSH $\beta$ -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 TSH $\beta$  mRNA levels

290 throughout metamorphosis parallel those observed in TSH cells using  
291 immunohistochemical methods; TSH $\beta$  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 TSH $\beta$ -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 TSH $\beta$ -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 TSH $\beta$ -IR cells first appear. In  
310 addition, TSH $\beta$ -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 TSH $\beta$ -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 TSH $\beta$ -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 TSH $\beta$ -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 TSH $\beta$ -IR during the late stages of embryogenesis. This may  
343 indicate that TSH $\beta$  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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361

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490 **FIGURE LEGENDS**

491 Fig. 1. Sagittal sections (6  $\mu\text{m}$ ) through the median eminence-pituitary of *E. coqui*  
492 embryos stained with Alcian blue, direct red, celestine blue and hematoxylin (Hall,  
493 1985). Anterior is to the left. (A) Townsend and Stewart (TS; 1985) stage 8, (B)  
494 stage 10, (C) stage 12, (D) stage 14. Arrowheads: columnar epithelia forming  
495 ependymal cell layer. Large open arrows: internal zone formed of nerve fibers  
496 and lacking nuclei. Small arrows: capillaries characteristic of the external zone.  
497 Abbreviations: III, third ventricle; Pit, pituitary; PN, pars nervosa. Scale bars: 10  
498  $\mu\text{m}$ .

499 Fig. 2. Sagittal sections (6  $\mu\text{m}$ ) through the pituitary of *E. coqui* embryos at TS  
500 stage 10. Anterior is to the left. (A) Control section stained with human TSH $\beta$   
501 antibody preabsorbed with ovine TSH. No specific staining is apparent. (B)  
502 Section immunostained with human TSH $\beta$ . TSH $\beta$ -positive cells (arrowheads)  
503 appear dark. Abbreviations: III, third ventricle; Pit, pituitary. Scale bars: 10  $\mu\text{m}$ .

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

508 Fig. 4. Comparison of thyroid-axis formation in metamorphic frogs (left) and  
509 direct-developing *E. coqui* (right). (A) Different life-history strategies are  
510 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 Gallery and Elinson (2000), Jennings and Hanken  
524 (1998), and this study.