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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

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19

20   **ABSTRACT**

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

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.

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 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

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 hr 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 hr. 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-

152   image. Total pituitary area and the area of TSHβ-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 TSHβ-IR cell volume were calculated by

155   multiplying area measurements by section thickness. Percent TSHβ-IR cell

156   volume for each specimen was calculated by dividing TSHβ-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 TSHβ 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 TSHβ 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 TSHβ (Fig 2A).

184

185 **3.2.2 Localization and ontogeny of TSHβ immunoreactive cells in the**

186 **pituitary**

187 Cells immunoreactive to rabbit anti-human TSHβ 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 TSHβ. 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 TSHβ-IR cell**

195 **volume**

196 Quantitative measurements of pituitary volume, TSHβ-IR volume, and percent

197 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

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 adrenocorticotropic 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,* TSHβ-IR cells are located in the mid-ventral portion of the pituitary. A

265 similar distribution of TSHβ-IR cells is seen by using antibodies to hTSHβ 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 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).

14

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

16

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361

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FIGURE LEGENDS

491 Fig. 1. Sagittal sections (6 μm) 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 μm.

499 Fig. 2. Sagittal sections (6 μm) through the pituitary of *E. coqui* embryos at TS

500 stage 10. Anterior is to the left. (A) Control section stained with human TSHβ

501 antibody preabsorbed with ovine TSH. No specific staining is apparent. (B)

502 Section immunostained with human TSHβ. TSHβ-positive cells (arrowheads)

503 appear dark. Abbreviations: III, third ventricle; Pit, pituitary. Scale bars: 10 μm.

504 Fig. 3. Histograms of pituitary and TSHβ measurements. Each bar represents the

505 mean of five specimens +/- 1 SEM. Groups sharing superscripts are not

506 significantly different (Tukey-HSD, $p > 0.05$). (A) Pituitary volume, (B) volume of

507 TSHβ-immunoreactive cells, (C) percent THSβ 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 Callery and Elinson (2000), Jennings and Hanken

524   (1998), and this study.