Ontogeny of the Thyroid Glands During Larval Development of South American Horned Frogs (Anura, Ceratophryidae)

Marissa Fabrezi & Julio César Cruz

Evolutionary Biology Evolutionary Biology

ISSN 0071-3260

Evol Biol DOI 10.1007/s11692-014-9292-5





Your article is protected by copyright and all rights are held exclusively by Springer Science +Business Media New York. This e-offprint is for personal use only and shall not be selfarchived in electronic repositories. If you wish to self-archive your article, please use the accepted manuscript version for posting on your own website. You may further deposit the accepted manuscript version in any repository, provided it is only made publicly available 12 months after official publication or later and provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The final publication is available at link.springer.com".



RESEARCH ARTICLE

Ontogeny of the Thyroid Glands During Larval Development of South American Horned Frogs (Anura, Ceratophryidae)

Marissa Fabrezi · Julio César Cruz

Received: 16 April 2014/Accepted: 3 August 2014 © Springer Science+Business Media New York 2014

Abstract The role of thyroid hormone (TH) in anuran metamorphosis has been documented from a variety of approaches, but the sequence of morpho-histological development of the thyroid glands that produce the secretion of the hormone was assumed invariant from studies of relatively few species even when the effects of environmental influences on larval development and metamorphosis have been largely documented. There are anurans in which developmental and growth rates diverge, and the resulting heterochrony in growth and development produces giant/miniature tadpoles, and or rapid/delayed metamorphosis suggesting changes of the activity of the thyroid glands during larval development. Herein, we analyze the morpho-histological variation of the thyroid glands in larval series of Ceratophrys cranwelli, Chacophrys pierottii, Lepidobatrachus laevis and L. llanensis that share breeding sites along semiarid environments of the Chaco in South America, belong to a monophyletic lineage, and present accelerated patterns in growth and development in order to have a morphological evidence about a possible shift of TH physiology. We describe gross morphology and histology of the thyroid glands and find features shared by all studied species such as the presence of supernumerary heterotopic follicles; changes in the volume and number of follicles towards the metamorphic climax, and cuboidal epithelia with occasional intra-cellular vacuoles as signs of low glandular activity without a manifest peak at the climax as it was assumed for anurans. We discuss different lines of evidence to interpret sources of extra supplement of TH to support the rapid

M. Fabrezi (🖂) · J. C. Cruz

Centro Científico Tecnológico-Salta, Instituto de Bio y Geociencias, CONICET, Salta, Argentina e-mail: mfabrezi@aol.com metamorphosis. These interpretations highlight the necessity to design a research program to investigate the endocrine variation during development of ceratophryids taking in account their morphology, physiology and ecology in order to learn more about the effects of environmental and developmental interactions involved in the anuran evolution.

Introduction

At present, most investigations of the amphibian thyroid are focused on the role of thyroid hormone (TH) in promoting the events of anuran metamorphosis, with studies analyzing TH levels, expression patterns of TH receptors (TR), the role of TR, the actions of TH on organogenesis, and the mechanisms that underlie the pleiotropic actions of THs (Callery and Elinson 2000; Fernandez-Mongil et al. 2009; Fort et al. 2007; Huang et al. 2001; Opitz et al. 2006; Schreiber et al. 2005; among others). From a more broadly biological perspective, there are two major generalizations that have emerged about the relationships between TH, thyroid glands, and amphibian development:

1. Amphibian metamorphosis is mediated directly by the thyroid gland via TH and indirectly by the pars distalis of the pituitary, via its thyroid-stimulating hormone (TSH). Metamorphosis cannot occur if either gland is absent or malfunctional (Dodd and Dodd 1976; Rot-Nikcevic and Wassersug 2003, 2004). At the time of the metamorphosis in anurans, in response to (external and/or internal) stimuli, the hypothalamus increases

the production of corticotropin-releasing-factor (CRF) that acts on pituitary gland to stimulate the release of TSH and adrenocorticotropic hormone (ACTH). Both hormones act on thyroid and adrenal glands respectively, increasing levels of TH and corticosterone (CORT). The corticosterone acts as synergist with TH to enhance the sensitivity of tissues to TH (Denver 2013).

2. The ontogeny of the thyroid gland in relation to metamorphosis is essentially the same in all anurans. This view dates back to Etkin (1936), who described the main changes in size and histology of the thyroid glands during the larval development of Lithobates palustris, L. catesbeianus and Pseudacris triseriata. He distinguished the following phases: premetamorphosis, in which the gland is small, and its cells are poor in cytoplasm; prometamorphosis, characterized by hind limb growth, when the thyroid shows a marked acceleration of growth and the follicular cell height increments to becomes columnar at forelimb emergence; and metamorphic climax, the period in which the epithelium becomes lower almost to the squamous condition at the end of the period and the follicles collapse. A similar pattern of changes during development has been described for other anuran taxa [e.g. Rana temporaria (Fox 1966); Bufotes viridis, Pelophylax ridibundus and Hyla savignyi (Michael and Al Adhami 1974); Rhinella arenarum (Miranda and Pisanó 1996), Xenopus laevis (Dodd and Dodd 1976; Grim et al. 2009) and the direct developer Eleutherodactylus coqui (Jennings and Hanken 1998), among others]. Some studies on TH physiology in anurans have assumed the absence of TH before differentiation of thyroid glands (Gomez-Mestre et al. 2013; Hollar et al. 2011) although it has been described the presence of TH prior to thyroid gland differentiation in Rhinella marina (Weber et al. 1994) and deiodinase activity and TH as essential to early embryogenesis in X. laevis (Morvan Dubois et al. 2014).

Even if these generalizations provide the biological basis for molecular analyses of frog metamorphosis, they relate poorly to the regulatory mechanisms that are generally assumed by ecologists to control how tadpoles respond to environmental variation (Rose 2005). The best example on the interactions between environmental conditions and endocrine mechanisms influencing the life cycles are the studies focused on larval development of the North American spadefoot toads: Ledón-Rettig and Pfennig (2012) reconstructed divergent evolutionary events related to TH physiology, corticosteroids, diet, predatormediated behavior, and polymorphisms within the populations of different species of these toads; and Buchholz and Hayes (2005) have proposed a model for the evolution of rapid metamorphosis in which selection for short larval periods to avoid desiccation, especially in *Scaphiopus*, acted via changes in TH physiology by accelerated development of all tissues except the gonads, which differentiated at their ancestral rate.

Data from 18 species with biphasic life cycles that breed simultaneously in ephemeral ponds in semi-arid environments of the Chaco in South America demonstrated the larval period varies within limits in which developmental rate and growth rate usually are parallel; e.g. those species that achieve metamorphosis at a small size develop faster than those that reach the metamorphosis at a large size under similar ecological conditions (Fabrezi 2011). However, there are anurans in which developmental and growth rates diverge, and the resulting heterochrony in growth and development produces giant/miniature tadpoles, and or rapid/delayed metamorphosis (Fabrezi 2011; Fabrezi and Quinzio 2008). Among the studied species, growth heterochrony may occur without producing alterations in development (e.g. Chacophrys pierottii and Ceratophrys cranwelli) but it seems to be necessary or pre-required to produce distinctive dissociated patterns of larval development as occurs during the larval development in Lepidobatrachus spp. with individuals that reach large size when tail is lost (hypermorphosis) by high growth rate (acceleration); transformations from larval to adult plans are quite absent suggesting an early onset of metamorphic or adultlike morphologies with consequences in the adult body plan (Fabrezi 2011).

Frog genera Lepidobatrachus (three species), Chacophrys (one species), and Ceratophrys (eight species) are included in Ceratophryidae (Fig. 1). Lepidobatrachus spp. Ch. pierottii, and C. cranwelli are endemic and sympatric frogs of the semiarid Chaco in South America. Patterns of larval growth and development in these frogs vary even when they may breed together in the same temporary pond (Fig. 2). The uniqueness of the ontogeny of Lepidobatrachus indicates that evolution of anuran larval development may occasionally involve mid-metamorphic morphologies conserving a free feeding tadpole and reduction of the morphological-ecological differences between tadpoles and adults (Bloom et al. 2013; Carroll et al. 1991; Fabrezi 2011; Fabrezi and Lobo 2009; Fabrezi and Quinzio 2008; Hanken 1993; Quinzio and Fabrezi 2014) suggesting that embryos or larvae would have higher TH levels, increased TR availability or increased TR sensitivity Bloom et al. 2013). It thus might be expected that the thyroid glands in Lepidobatrachus tadpoles show an earlier onset of the peak of secretory activity.

Herein, we describe the morphological and histological changes of the thyroid glands in larval series of field captured specimens of *Chacophrys pierottii, Ceratophrys*



b Pyron and Wiens, 2011

Fig. 1 The most recent neobatrachian phylogenies, showing the major lineages diversified in South America. **a** Hypothesis by Frost et al. (2006) recovered a Ceratophryidae formed by *Atelognathus, Batrachyla, Ceratophrys, Chacophrys, Lepidobatrachus* and *Telmatobius*. The Ceratophryinae (*Ceratophrys, Chacophrys, Lepidobatrachus*) are found in tropical lowland South America from Colombia and Venezuela south to Argentina, whereas the other genera have a Southern Andean distribution extending to the extreme south of Argentina and Chile. **b** Hypothesis by Pyron and Wiens (2011) proposed a Ceratophryidae composed of *Ceratophrys, Chacophrys, Lepidobatrachus*, although *Ceratophrys* was found to be paraphyletic. Stars indicate those lineages that have endemisms to the Chaco. The Chaco comprises a plain of approximately 1,141,000 km² in the south-central South America (from 16°550S–33°520 and 67°500W–

57°520W). It is an arid and subtropical region of low forests and savannas traversed by only two permanent rivers: Pilcomayo and Bermejo that flow southeastward across the plain from their Andean headwaters to Paraguay River. The Chaco is subject to climates that vary from tropical in the north to warm-temperate in the south. The mean of annual temperature varies between 26° and 16° with an average of 22 °C. The highest recorded temperatures for the continent occur in the Chaco with absolute maximums that may reach 54 °C during December–January. There is marked seasonality, with warm summers with precipitations ranging with a West-East gradient from 300 to 1,000 mm. Evaporation losses (1,500–900 mm) sharply reduce the effective precipitation and give the Chaco an arid nature that is absent only in the permanent swamps and forests along Paraguay River

Author's personal copy



cranwelli, Lepidobatrachus laevis and *L. llanensis* with the goal being to look for any deviation from Etkin's (1936) generalized model for anuran thyroid development. The analysis of complete larval series with additional information from field data offers the opportunity to have the first approach on the endocrine features in frogs in which larval morphology and larval ecology seem to have

evolved in unique and unexpected direction learning more about this terrific group.

Besides, we hope this study highlights the importance of providing morphological, ecological and historical contexts to add information to those molecular and ultrastructural studies of anuran development since a full understanding of developmental plasticity, requires more than the **◄ Fig. 2 a** Graphs that show larval stage versus size during larval development in ceratophryids. The upper curve refers to TL (tail length + snout-vent length) with a change of slope towards prometamorphosis that implies an allometric growth of the tail with respect to the snout-vent length. At the end of premetamorphosis tadpoles have reached at least 60 % of the snout-vent length (lower curve) at metamorphosis. C. cranwelli exhibits the least growth among the studied species. b Graphs that show days of development versus size during larval development in ceratophryids. Lepidobatrachus spp. and Ch. pierottii develop in 2 weeks, faster than C. cranwelli that metamorphoses after 21 days of development. Further, growth in Lepidobatrachus spp. and Ch. pierottii increases intensively in a shorter period of time. These graphs reveal differences between the developmental and growth rates within the ceratophryids. Accelerated rates in Lepidobatrachus spp. and Ch. pierottii with respect to C. cranwelli or retarded rate in C. cranwelli with respect to Lepidobatrachus spp. and Ch. pierottii. Comparisons with other anurans that share similar breeding sites demonstrate that the ceratophryids generally present accelerated rates of growth and development (Fabrezi 2011). The monophyly of the group assures that observed variation among species will reflect evolutionary changes. Despite the fact that these ceratophryids are sympatric and share similar environmental requirements, their larvae differ in morphology and ecology: Ceratophrys and Lepidobatrachus have macrophagous and specialized carnivorous larvae (Ruibal and Thomas 1988; Wassersug and Heyer 1988), whereas the tadpole of Ch. pierottii (Faivovich and Carrizo1992; Quinzio et al. 2006) may be characterized as a typical pond-type tadpole with generalized suspension feeding (Duellman and Trueb 1986). Besides, in Lepidobatrachus spp. the peramorphic larval body plan that is insinuated into the free feeding stage is likely equivalent to the metamorphic larval stages (between forelimb emergence and complete tail loss) of most anurans, suggesting that their climax is different (Fabrezi 2011; Fabrezi and Quinzio 2008; Hanken 1993)

identification of the mechanisms and pathways by which individual factors affect tadpole growth and development (Rose 2005).

Materials and Methods

This study analyzed histo-morphological variation of the thyroid glands in larval and postmetamorphic specimens of C. cranwelli, Ch. pierottii, L.laevis and L. llanensis (Figs. 1, 2). These specimens were collected between November and April 1991–2011 in ephemeral ponds along Ruta provincial N°5 (24°43'S, 64°11'W) in Departamento Anta, Ruta Nacional 81 (23°81'S, 63°83'W) in Departamento San Martín, Provincia de Salta (Argentina), and on January 2006 at Totoralejos (29°37'60"S, 64°50'60"W) in northwestern Córdoba (Argentina). Specimens were fixed in 10 % formalin in the field. Lots of specimens are deposited in the Herpetological Collection of the Museo de Ciencias Naturales (MCN-IBIGEO, CONICET and Universidad Nacional de Salta) Argentina, with the following collection numbers: C. cranwelli (021, 421, 413, 425, 607, 670, 1.318); Ch. pierottii (987, 1.028, 1.029, 1.030), L. laevis (647, 663, 695, 1.320), and L. llanensis (567, 605,

665, 666, 1.319). Additional larval specimens at prometamorphic and climax stages of *Rhinella schneideri* (1.316) from Ruta provincial N°5 (24°43′S, 64°11′W) in Departamento Anta, Provincia de Salta (Argentina) were used for comparisons.

Larval series were obtained following the standard table of Gosner (1960), Fabrezi and Quinzio (2008) for advanced larval stages of *Lepidobatrachus* spp. We measured the body size (SVL) and total length (TL) for each specimen using dial calipers (0.02 mm) and measurements are given in millimeter (mm). Manual dissection allowed us to describe the gross morphology of the thyroid glands. The glands were observed by removing the throat skin, interhyoideus muscle, and subarcualis obliquus muscles (Fig. 3). Descriptions and illustrations of the glands were made with a Nikon-SMZ1000 stereo dissection microscope equipped with a Nikon Coolpix digital camera.

In each species, individuals at different larval stages representing premetamorphosis (27–35), prometamorphosis (36–41), and climax (42–46), and one postmetamorphic specimen of *L. laevis*, were selected for histological preparations. Then, the buccal floor containing the hyobranchial apparatus was separated, dehydrated in ascending ethanol series (50–100 %), cleared in xylene, embedded in paraffin, and sectioned at 4 μ m with a Leica RM 2245 rotatory semiautomatic microtome. Sections were stained with hematoxylin and eosin following the protocol by Martoja and Martoja-Pierson (1970). Observations and illustrations were made with a Nikon E200 microscope, equipped with digital camera and Micrometrics software.

Additional specimens were dissected to add data on gut morphology and diet for the discussion.

Results

Gross Morphology

In *C. cranwelli* the thyroid glands were observed from tadpoles at Stage 30. Most of the examined specimens present a pair of glandular masses placed internal and contiguous to the origin of the m. geniohyoideus (Fig. 3a). Each gland is oblong, compressed dorso-ventrally, and the follicles are visible by their transparency. As development advances (Fig. 4a–c), the glands increase their volume slightly and become rounded although these changes are not well marked. During the climax stages they acquire a spherical configuration (Fig. 4d). The metamorphic transformations of the mm. geniohyoideus (duplication to give rise to the pars medialis and externa) and the mm. sub-arcualis obliquus (to turn into the mm. sternohyoideus) hide the glands, which in the metamorphosed frog are placed adjacent to the origin of the posteromedial processes



Fig. 3 Schematic representation of the topographical relationships of the thyroid glands within ceratophryids. **a** *C. cranwelli* larvae, view of hyobranchial apparatus. The thyroid glands are discovered when the mm. subarcualis obliquus are separated, they are internal and contiguous to the origin of the mm. geniohyoideus. **b** *Ch. pierottii* larvae, view of hyobranchial apparatus. As the mm. subarcualis obliquus are thin and bear long tendons, the thyroid glands are seen without the necessity to remove them. **c** *Lepidobatrachus* spp. larvae; the glands are internal and anterior to the origin of the mm. geniohyoideus. **d** The thyroid glands in adults of *C. cranwelli* and *Ch. pierottii* are attached to the ventral face of the hyoid plate, at the base of the posterolateral processes. The mm. geniohyoideus lateralis and

of the hyoid and between the mm. geniohyoideus medialis and geniohyoideus lateralis (Fig. 3d). Some larval specimens may present secondary follicular masses (Fig. 4c, d).

In *Ch. pierottii* the thyroid glands were first observed in tadpoles at Stage 27, with a pair of masses placed internally and anteriorly to the origin of the m. geniohyoideus (Fig. 3b). During premetamorphosis each mass is compact and cordiform with numerous small follicles that are seen by their transparency (Fig. 4e). During prometamorphosis the glands increase their volume, become longer, and lose the cordiform shape (Fig. 4f, g). At the end of the prometamorphosis the glands are cylindrical, conserving this shape up to the end of metamorphosis (Fig. 4h). In metamorphosed individuals the glands have similar topographic relationships with the muscles as was described in *C. cranwelli* (Fig. 3d). Some larval specimens may present a secondary follicular mass medially and anteriorly, attached to the m. geniohyoideus (Fig. 4f).

In earlier larval stages of *Lepidobatrachus* spp. (Stage 28) the incipient thyroid glands are observed adjacent to

geniohyoideus medialis run on either side of each gland, and the mm. sternohyoideus pars ventralis and sternohyoideus pars dorsalis cover the gland ventrally. **e** The thyroid glands in adults of *L. llanensis* are attached to the ventral face of the hyoid plate, at the base of the posterolateral processes. The mm. geniohyoideus lateralis and geniohyoideus medialis run along the sides of each gland, and the mm. sternohyoideus pars ventralis and sternohyoideus pars dorsalis cover the gland ventrally. **f** The thyroid glands in adults of *L. laevis* are attached to the ventral face of the hyoid plate, posteriorly at the basis of the posterolateral processes. The m. geniohyoideus medialis runs internally to each gland, and the mm. sternohyoideus pars ventralis and sternohyoideus pars ventralis and sternohyoideus pars ventralis runs internally to each gland, and the mm. sternohyoideus pars ventralis and sternohyoideus pars ventralis and sternohyoideus pars ventralis and sternohyoideus pars ventralis and the mm. sternohyoideus pars ventralis and sternohyoideus pars ventralis runs internally to each gland, and the mm. sternohyoideus pars ventralis and sternohyoideus pars dorsalis cover the gland ventrally.

the processus urobranchialis (Fig. 3c). Each primary gland is formed by aggregation of groups of unorganized follicles and the lumens are not yet defined. The shape and size of each gland varies both within and among individuals. During premetamorphosis the glands grow (Fig. 4i, j, o, p). The follicles are small and numerous and the lumens are observed by their transparency. During prometamorphosis, the presence of primary (those placed adjacent the processus urobranchialis) and secondary groups of thyroid follicles differs among the individuals. Secondary groups of follicles have different shapes, sizes and location and some times, the primary groups of follicles are long and may present constrictions that suggest the glandular groups are dividing or fusing (Fig. 4k-m, q-s). During early climax stages the glands (primary and secondary masses) grow and the lumens of the follicles seem to be larger than in prometamorphic stages (Fig. 4n, t). At the end of climax, each gland (presumably derived from the primary pair) is an elongate mass attached the hyoid plate, placed between the mm, geniohyoideus lateralis and geniohyoideus

Author's personal copy



Fig. 4 Thyroid glands during larval development in *C. cranwelli* (\mathbf{a} - \mathbf{d} scale bar = 0.5 mm); *Ch. pierottii* (\mathbf{e} - \mathbf{h} scale bar = 0.5 mm); *L. laevis* (\mathbf{i} - \mathbf{n} scale bar = 0.1 mm) and *L. llanensis* (\mathbf{o} - \mathbf{t} scale bar = 0.5 mm). Note the presence of ectopic follicles

medialis in *L. llanensis* (Fig. 3e) and external to the m. geniohyoideus medialis in *L. laevis* (Figs. 3f). Each gland is hidden by the mm. sternohyoideus (pars dorsalis and ventralis).

Histology

The histology of the thyroid gland of *C. cranwelli* reveals the gland has a triangular contour in transverse section (Fig. 5a). The follicular cells are grouped in central follicles surrounded by small and incipient follicles. The follicular cells form a low cuboidal epithelium. The nuclei of the follicular cells are condensed and have oval or rounded shapes. The cytoplasm of these cells is acidophilic, scarce and homogeneous. At later stages of premetamorphosis there is a small amount of colloid in the follicular lumens (Fig. 6a). During prometamorphosis the gland is dorsoventrally compressed in transverse section (Fig. 6b). The follicular cells initially have condensed and central nuclei that change to a more basal position with less condensed chromatin. The epithelium of the follicles is cuboidal and higher that previous stages and becomes columnar towards the climax stages. The increase in the height of the follicular cells is due to the presence of numerous intra-cellular vacuoles in the apical cytoplasm. The presence of eosinophilic colloid within the follicular lumens is detected during the prometamorphosis (Fig. 6b) and is remarkable during the climax stages 44–45 (Fig. 6c). The height of the follicular epithelium decreases progressively to cuboidal and coincides with the collapse of the follicles at the end of the climax (Fig. 6d). The contour of the gland becomes dorso-ventrally flattened again.

The thyroid glands in *Ch. pierottii* have numerous follicles better defined at the end of the premetamorphosis and have circular contour (Fig. 5b). During premetamorphosis the follicular epithelium is cuboidal, the cytoplasm of the follicular cells is homogeneous and the nuclei are spherical. The lumens of the glands do not present colloid (Fig. 6e). At prometamorphic stages the thyroid glands exhibit a progressive increase in the number of follicles.



Fig. 5 Cross section of the thyroid gland and its relation with the hypobranchial skeleton and musculature at larval Stage 37. a *C. cranwelli*, b *Ch. pierotti*, c *L. laevis*, and d *L. llanensis. Ch. pierottii*

has the largest glands within the studied species. hb hypobranchial cartilage, tg thyroid gland, mgh muscle geniohyoideus. Scale bar = 0.5 mm

The epithelium of the follicles is always cuboidal and later stages show only a few follicles with colloid (Fig. 6f). In subsequent developmental stages the follicles have a cuboidal epithelium. Follicular cells have slightly condensed nuclei and apical vacuoles in the cytoplasm (Fig. 6g). In late climax the epithelium decreases progressively in height to become low cuboidal as well as decreases the amount of cytoplasmic vacuoles. Further, the nuclei are ovoid, basophilic and strongly condensed (Fig. 6h).

Lepidobatrachus laevis and L. llanensis share similar histological features. During earlier premetamorphic stages the gland consists only of some cells grouped irregularly or a few incipient follicles with low cuboidal epithelium without colloid. Follicular cells have condensed and ovoid or spherical nuclei. The acidophilic cytoplasm is scarce and homogeneous (Fig. 6i). Towards the end of this period and into prometamorphic stages there is a progressive increase in size of the gland due to the growth in the number and size of follicles (Figs. 5c, d, 6j). The epithelium is mostly cuboidal and the apical cytoplasm of follicular cells contains vacuoles. These vacuoles increase progressively in number to the end of the prometamorphosis. The follicular cells are uniformly cuboidal with basal and spherical nuclei. During the prometamorphosis the follicular epithelium reaches its maximum height (Fig. 6j). Colloid accumulation was never observed. At climax stages the follicular lumens are large and round. The epithelium has cuboidal follicular cells with less intra-cellular vacuoles (Fig. 6k). These cells have basal and less condensed nuclei at the beginning of the climax. At the end of climax stages most of the follicular lumens collapse; cells of the epithelium become low cuboidal without vacuoles and their well-condensed nuclei are located centrally. Again, colloid accumulation was not observed (Fig. 6l).

The thyroid glands in a postmetamorphic individual of *L. laevis* are dorso-ventrally flattened, as are the follicular lumens. The follicular lumens are filled with acidophilic colloid. The epithelium of the follicles is flat and its cells have large and condensed nuclei with scarce cytoplasm (Fig. 7a).

Author's personal copy



Fig. 6 Higher-magnification images of the normal microanatomy of thyroid glands during premetamorphosis (Gosner Stages 26–35), prometamorphosis (Gosner Stages 36–41) and climax (Gosner Stages 42–46) of ceratophryids. **a–d** *C. cranwelli;* **e–h** *Ch. pierottii* 1. *L. laevis*, and **j–l** *L. llanensis*. Height of the cells increases at

Discussion

The development of the thyroid glands in Ceratophryidae presents some features that have not been described in any other taxa. At the level of gross morphology, the primary pair of glands may be accompanied by secondary groups of heterotopic follicles that appear in the throat musculature;

prometamorphic and early climax stages but they never become columnar. Colloid accumulation (*asterisk*) is clearly observed in *C. cranwelli* from premetamorphosis on, whereas in *Ch. pierottii* a low amount appears only at the end of prometamorphosis. *Scale* $bar = 50 \ \mu m$

this fact is more frequent, marked, and earlier within *Lepidobatrachus* spp. Furthermore, the glands in Ceratophryidae have more numerous and smaller follicles than other stereotypic taxa (Fig. 7b).

Heterotopic thyroid follicles have been mentioned in bony fishes (Norris and Carr 2013) and metamorphic individuals of the large salamander *Pleurodeles waltl* (Gozzo



Fig. 7 a Cross sections of the thyroid gland in a postmetamorphic individual of *L. laevis* in which the tympanic membrane is already differentiated. The transverse section denotes the flattened contour of the gland that is consequence of the collapse of follicles. The gland seems to be inactive and the presence of colloid suggests lower level of secretion (Brande-Lavridsen et al. 2010). *Scale bar* = 200 μ m. **b** Cross sections of the thyroid gland in a specimen of *Rhinella schneideri* at advanced prometamorphic stage (Gosner Stage 40). The follicles are large with columnar epithelia. The cells have basal nuclei and apical vacuoles. This image reveals the secretory activity of the thyroid glands is increased to mediate the metamorphic transformations as was described by Etkin (1936). *Scale bar* = 30 μ m

et al. 1996). The occurrence of accessory thyroid follicles in the throat musculature has been attributed to the absence of connective tissue delimiting the glands (Norris and Carr 2013). Dodd and Dodd (1976) demonstrated that *X. laevis* tadpoles thyroidectomized at larval Stage 58 (Nieuwkoop and Faber Table) are able to regenerate thyroid tissue with remarkable speed from supernumerary follicles or remnants of follicles adhering to the thyroid artery; enough thyroid tissue to mediate climax can be regenerated in 10 days.

The increase of the epithelial cell height, depletion of the colloid and appearance of intra-cellular vacuoles, as well as decrease in follicular cell number and size of follicles are responses to increasing TH levels in blood, whereas increased follicular cell height is used as a morphological indicator of TSH response (Brande-Lavridsen et al. 2010; Dodd and Dodd 1976; Etkin 1936; and Fig. 7). Through our histological studies we found the thyroid glands do not present the generalized characteristics described by Etkin (1936), since their epithelia never become high cuboidal to high columnar with abundant intra-cellular vacuoles, features indicating that the maximum peak of activity of the glands has been reached (Fig. 7b). Moreover, the lumens are empty in *Ch. pierottii* and *Lepidobatrachus* spp. This fact does not agree with our initial supposition that the ceratophryids would show an earlier onset of the peak of glandular activity associated with their rapid development and metamorphosis.

We will discuss possible explanations and propose new questions.

Presence of Endogenous TH in Absence of Thyroid Glands

Weber et al. (1994) found T_3 and T_4 prior differentiation of the thyroid glands in *Rhinella marina*. The presence of these hormones was detected as early as blastopore formation and suggested they are probably maternal origin. Morvan Dubois et al. (2014) described deiodinase expression in neurogenic areas both in the central and peripheral nervous system in *X. laevis* embryos demonstrating the TH signally (deiodinases control levels of T_3 and T_4 available to different tissues) is a key component of early neuronal development in vertebrates.

Studies on GNM (giant non-metamorphosing) tadpoles have demonstrated that the absence of thyroid glands (and TH) could preclude the metamorphosis and then, larval growth could continue indefinitely; these tadpoles may reach 1-8 years old although normal thyroid signaling advances in head, axial, and limb skeletons (Rot-Nikcevic and Wassersug, 2003, 2004; Kerney et al., 2009). Smirnov and Vassilieva (2014) compared X. laevis "giant tadpoles" described in literature and goitrogen arrested tadpoles and found they differ in that they have features which require TH to appear suggesting the appearance of TH depended features in giant tadpoles would indicate the occurrence of the additional sources of TH. As GNM tadpoles are advanced in age it would be impossible they retain maternal TH deposited in the oocytes, then the occurrence of heterotopic thyroid follicles situated beyond a thyroid gland is proposed (Smirnov and Vassilieva 2014).

Presence of Exogenous TH in Presence of Thyroid Glands

Kupferberg (1997) discussed several lines of evidence indicating that increased protein in the larval diet and

cannibalism enhances both growth and development, and concluded food resources can influence anuran metamorphosis, and diet may induce changes in thyroid function.

A good example of this is found in North American spadefoot toads of the genera Spea and Scaphiopus. As Spea and Scaphiopus are sister clades, the evolution of some ecological and physiological trends (TH physiology, corticosteroids, diet, predator-mediated behavior, polymorphisms, desiccation, temperature) provides a unique opportunity to reconstruct evolutionary events and to compare with other anuran lineages. Spea multiplicata may rapidly develop into large, carnivorous morphs and into smaller, slowly-developing omnivorous morphs (Pfennig 1992a). Exposure to exogenous thyroid produces carnivorous morphs, and the ingestion of other tadpoles with T_4 or of shrimp with T₂ could be the mechanism to stimulate the development of carnivorous morphs (Pfennig 1992b). Scaphiopus bombifrons presents larvae that feed on detritus that develop into herbivorous omnivores, while those that fed on fairy shrimp become carnivores that are cannibalistic (Pfennig et al. 1993). The existence of some Scaphiopus couchii populations that are sympatric and others allopatric with Spea reveals that the presence of carnivorous Spea tadpoles causes S. couchii to lose the ability to consume shrimp. This suggests that, originally, both Spea and *Scaphiopus* tadpoles consumed anostracan shrimp; over time, Spea tadpoles evolved polyphenisms that enhanced their ability to consume shrimp or tadpoles, shrimp-fed Scaphiopus larvae experienced reduced growth and developmental rates, as well as elevated levels of the stress hormone corticosterone when compared with those that ate the ancestral detritus diet (Ledón-Rettig and Pfennig 2012; Ledón-Rettig et al. 2009). Other experimental studies on TH physiology (TH content and/or stimulation, TR α and TR β gene expression, oxidative stress enzyme activity; corticosterone) varying environmental parameters (water level and temperature) demonstrated the effect of water level in the speed of development and some morphological traits (size at metamorphosis, limb length, and accumulation of fat body content, etc.) in the spadefoots and proposed a model for the evolution of rapid metamorphosis in which selection for short larval periods to avoid desiccation in Scaphiopus, acted via changes in TH physiology by the augment of TH sensitivity in tail tissues (Buchholz and Hayes 2005; Gomez-Mestre and Buchholz 2006; Gomez-Mestre et al. 2013; Hollar et al. 2011; Kulkarni et al. 2011). However a recent paper by Zeng et al. (2014) tested this last hypothesis in a phylogenetic framework and found rapid development in pelobatoids is strongly related to their small genome size.

Larval growth in ceratophryids is fast, which may be a consequence of the warm, semitropical bodies of water

with few predators that provide ideal conditions to breed giant tadpoles (Roçek et al. 2006, Fig. 1). Rapid development and growth also enhances the chances of avoiding predators, and provide the opportunity to consume other animals. The ceratophryid genera have different larval morphotypes in which the feeding mechanism, gut and diet varies: Chacophrys is a suspension feeder and omnivore with a long and spiraled gut completely occupied with some algae, macerated soft food (shrimps) mixed with detritus. C. cranwelli has strong keratinized jaw sheaths that permit tearing the prey, and presents a short intestine divided into an anterior dilated segment and a posterior spiral intestine; the foregut may contain tadpoles, shrimps, insects and insect larvae, and the hind gut bears feces. Lepidobatrachus spp. have wide mouths that allow them to swallow the prey whole, a well differentiated stomach with pepsin secretion and a short intestine, the stomach usually is fully occupied by heterospecific tadpoles, shrimps and con-specific tadpoles (Bloom et al. 2013; Carroll et al. 1991; Fabrezi 2011). Furthermore, Lepidobatrachus spp. and C. cranwelli tadpoles are voracious predators in which cannibalism is frequent. Lepidobatrachus tadpoles and post metamorphic individuals remain in the aquatic habitat, feed underwater, being voracious predators on insects and other tadpoles, including co-specific ones, so desiccation would be a factor without influences in their development and metamorphosis.

The fact that during development the ceratophryids show thyroid glands with discrete glandular activity (synthesis and colloid accumulation) is consistent with different lines of evidence: (1) ceratophryids have eggs range in size 1.8-2.5 mm. This size could mean the accumulation of maternal molecules including T₃ and T₄ enough to supply and accelerate growth and the development without producing a peak of activity of thyroid glands; (2) the proliferation of heterotopic follicles multiply the secretory tissue to mediate rapidly the climax metamorphic; and (3) the ceratophryids are able to grow and develop fast by receive extra exogenous TH or TH precursors from diet. These possible explanations denote the necessity to design a research program to investigate the endocrine variation during development of ceratophryids since these nonmodel species-that seem to have evolved with a unique spatial and temporal history-represent the opportunity to learn more about the effects of reciprocal environmental and developmental interactions involved in the anuran evolution. Most of the knowledge about the role of the thyroid gland in amphibian metamorphosis has been obtained without a broad biological perspective, taking into account the diversity of life history modes in anurans. Despite the major contributions to EVO DEVO proceeding from advances in developmental genetics and experimental studies on model species, a better comprehension of the evolution of phenotypes still needs further data from comparative embryology, morphology and systematics in non-model species.

Before ending, we must remark on the fact that in reviewing the literature for this research, we failed to find information on how most of the lab reared specimens from which the morphology and histology of thyroid glands were studied were fed. Also, we were unable to find any morphological and/or histological description of the thyroid glands in spadefoot larvae. These facts highlight the present difficulty in achieving and integrated understanding of the role of thyroid glands, TH, and nutrition in determining the timing of development in anurans.

Acknowledgments We thank two anonymous reviewers for helpful criticisms on the manuscript and figures; John Reiss provided careful comments on an earlier draft of the manuscript and many suggestions on this English written; Javier Goldberg and Silvia Quinzio contributed with observations for a constructive discussion; Secretaría de Medio Ambiente y Desarrollo Sustentable, Provincia de Salta gave permissions to collect the specimens of this study. This research was supported by CONICET: PIP 239 and Agencia Nacional de Promoción Científica y Tecnológica: PICT-Bicentenario 0616.

References

- Bloom, S., Ledón-Rettig, C., Infante, C., Everly, A., Hanken, J., & Nascone-Yoder, N. (2013). Developmental origins of a novel gut morphology in frogs. *Evolution and Development*, 15(3), 213–223.
- Brande-Lavridsen, N., Christensen-Dalsgaard, J., & Korsgaard, B. I. (2010). Effects of ethinylestradiol and the fungicide prochloraz on metamorphosis and thyroid gland morphology in *Rana temporaria*. *The Open Zoology Journal*, 2010(3), 7–16.
- Buchholz, D. R., & Hayes, T. (2005). Variation in thyroid hormone action and tissue content underlies species differences in the timing of metamorphosis in desert frogs. *Evolution and Devel*opment, 7(5), 458–467.
- Callery, E. M., & Elinson, R. P. (2000). Thyroid hormone-dependent metamorphosis in a direct developing frog. *Proceedings of the National Academy of Sciences*, 97(6), 2615–2620.
- Carroll, E. J., Seneviratne, A. M., & Ruibal, R. R. (1991). Gastric pepsin in an anuran larva. *Development, Growth & Differentiation*, 33(5), 499–507.
- Denver, R. J. (2013). Neuroendocrinology of amphibian metamorphosis. Current Topics in Developmental Biology, 103, 195–227.
- Dodd, M. H. I., & Dodd, J. M. (1976). The biology of metamorphosis. In B. Lofts (Ed.), *Physiology of the Amphibia* (pp. 467–599). New York: Academic Press Inc.
- Duellman, W. E., & Trueb, L. (1986). Biology of Amphibians. Baltimore: The Johns Hopkins University Press.
- Etkin, W. (1936). The phenomena of the anuran metamorphosis. III. The development of the thyroid gland. *Journal of Morphology*, *59*(1), 68–89.
- Fabrezi, M. (2011). Heterochrony in growth and development in anurans from the Chaco of South America. *Evolutionary Biology*, 38(4), 390–411.
- Fabrezi, M., & Lobo, F. J. (2009). Hyoid skeleton, related muscles, and morphological novelties in the frog *Lepidobatrachus* (Anura, Ceratophryidae). *The Anatomical Record*, 292, 1700–1712.

- Fabrezi, M., & Quinzio, S. I. (2008). Morphological evolution in Ceratophyinae frogs (Anura. Neobatrachia): The effects of heterochronic changes during larval development and metamorphosis. *Zoological Journal of the Linnean Society*, 2008(154), 752–780.
- Faivovich, J., & Carrizo, G. R. (1992). Descripción de la larva de *Chacophrys pierottii* (Vellard, 1948) (Leptodactylidae, Ceratophryinae). *Alytes*, 10(3), 81–89.
- Fernandez-Mongil, M., Venza, C. J., Rivera, A., Dominicci, J. A., Burggren, W., & Rojas, L. V. (2009). Triiodothyronine (T₃) action on aquatic locomotor behavior during metamorphosis of the bullfrog *Rana catesbeiana*. *The International Journal of Developmental Biology*, 53(1), 101–108.
- Fort, D. J., Degitz, S., Tietge, J., & Touart, L. W. (2007). The hypothalamic pituitary-thyroid (HPT) axis in frogs and its role in frog development and reproduction. *Critical Reviews in Toxicology*, 37, 117–161.
- Fox, H. (1966). Thyroid growth and its relationship to metamorphosis in *Rana temporaria*. Journal of Embryology and Experimental Morphology, 16(3), 487–496.
- Frost, D. R., Grant, T., Faivovich, J., Bain, R. H., Haas, A., Haddad, C. F. B., et al. (2006). The amphibian tree of life. *Bulletin of American Museum of Natural History*, 297, 1–370.
- Gomez-Mestre, I., & Buchholz, D. R. (2006). Developmental plasticity mirrors differences among taxa in spadefoot toads linking plasticity and diversity. *Proceedings of the National Academy of Sciences*, 103(5), 19021–19026.
- Gomez-Mestre, I., Kulkarni, S., & Buchholz, D. R. (2013). Mechanisms and consequences of developmental acceleration in tadpoles responding to pond drying. *PLoS ONE*, 8(12), e84266.
- Gosner, K. (1960). A simplified table for staging anurans embryos and larvae, with notes on identification. *Herpetologica*, 16(3), 183–190.
- Gozzo, S., Taglioni, A., Casetti, R., Meloni, M., Bagnoli, C., & Monaco, V. (1996). Supernumerary thyroid glands of *Pleurodeles waltl* (Caudata, Salamandridae). *Italian Journal of Zoology*, 63(3), 207–214.
- Grim, K. C., Wolfe, M., Braunbeck, T., Iguchi, T., Ohta, Y., Tooi, O., et al. (2009). Thyroid histopathology assessments for the amphibian metamorphosis assay to detect thyroid-active substances. *Toxicologic Pathology*, 37(4), 415–424.
- Hanken, J. (1993). Model systems versus outgroups: Alternative approaches to the study of head development and evolution. *American Zoologist*, 33(4), 448–456.
- Hollar, A. R., Choi, J., Grimm, A. T., & Buchholz, D. R. (2011). Higher thyroid hormone receptor expression correlates with short larval periods in spadefoot toads and increases metamorphic rate. *General and Comparative Endocrinology*, 173(2011), 190–198.
- Huang, H., Liquan, C., Remo, B. F., & Brown, D. D. (2001). Timing of metamorphosis and the onset of the negative feedback loop between the thyroid gland and the pituitary is controlled by type II iodothyronine deiodinase in *Xenopus laevis*. *Proceedings of the National Academy of Sciences*, 98(13), 7348–7353.
- Jennings, D. H., & Hanken, J. (1998). Mechanistic basis of life history evolution in anuran amphibians: thyroid gland development in the direct-developing frog, *Eleutherodactylus coqui. General* and Comparative Endocrinology, 111, 225–232.
- Kerney, R., Wassersug, R., & Hall, B. K. (2009). Skeletal advance and arrest in giant non-metamorphosing African clawed frog tadpoles (*Xenopus laevis*: Daudin). *Journal of Anatomy*, 2009(20), 1–12.
- Kulkarni, S. S., Gomez-Mestre, I., Moskalik, C. L., Storz, B. L., & Buchholz, D. R. (2011). Evolutionary reduction of developmental plasticity in desert spadefoot toads. *Journal of Evolutionary Biology*, 24(2011), 2445–2455.

- Kupferberg, S. (1997). The role of larval diet in anuran metamorphosis. American Zoologist, 37(2), 146–159.
- Ledón-Rettig, C. C., & Pfennig, D. W. (2012). Antipredator behavior promotes diversification of feeding strategies. *Integrative and Comparative Biology*, 52(1), 53–63.
- Ledón-Rettig, C. C., Pfennig, D. W., & Crespi, E. J. (2009). Stress hormones and the fitness consequences associated with the transition to a novel diet in larval amphibians. *Journal of Experimental Biology*, 212, 3743–3750.
- Martoja, R., & Martoja-Pierson, M. (1970). Técnicas de histología Animal. Barcelona: Toray-Masson.
- Michael, M. I., & Al Adhami, M. A. (1974). The development of the thyroid glands in anuran amphibians of Iraq. *Journal of Zoology*, 1974(174), 315–323.
- Miranda, L. A., & Pisanó, A. (1996). Estudio morfométrico sobre el desarrollo y evolución de las glándulas tiroides durante la metamorfosis de *Bufo arenarum. Cuadernos de herpetología*, 10(1–2), 47–57.
- Morvan Dubois, G., Sebillot, A., Kuiper, G. G. J. M., Verhoelst, C. H. J., Darras, V. M., Visser, T. J., et al. (2014). Deiodinase activity is present in *Xenopus laevis* during early embryogenesis. *Endocrinology*, 147(10), 4941–4949.
- Norris, D. O., & Carr, J. A. (2013). The hypothalamus-pituitaryethyroid (HPT) axis of non-mammalian vertebrates. In D. O. Norris & J. A. Carr (Eds.), *Vertebrate endocrinology* (pp. 231–257). Waltham: Academic Press.
- Opitz, R., Hartmann, S., Blank, T., Braunbeck, T., Lutz, I., & Kloas, W. (2006). Evaluation of histological and molecular endpoints for enhanced detection of thyroid system disruption in *Xenopus laevis* tadpoles. *Toxicological Sciences*, 90(2), 337–348.
- Pfennig, D. W. (1992). Polyphenism in spadefoot toad tadpoles as locally adjusted evolutionarily stable strategy. *Evolution*, 46(3), 1408–1420.
- Pfennig, D. W., Hudson, K. R., & Sherman, P. W. (1993). Kin recognition and cannibalism in spadefoot toad tadpoles. *Animal Behavior*, 1993(46), 87–94.
- Pfenning, D. W. (1992). Proximal and functional causes of pholyphenism in an anuran tadpole. *Functional Ecology*, 1992(6), 167–174.
- Pyron, R. A., & Wiens, J. J. (2011). A large-scale phylogeny of Amphibia including over 2,800 species, and a revised classification of extant frogs, salamanders, and caecilians. *Molecular Phylogenetics and Evolution*, 61(2), 543–583.

- Quinzio, S. I., & Fabrezi, M. (2014). The lateral line system in anuran tadpoles: Neuromast morphology, arrangement and innervation. *The Anatomical Record*. doi:10.1002/ar.22952.
- Quinzio, S., Fabrezi, M., & Faivovich, J. (2006). Redescription of the tadpole of *Chacophrys pierottii* (Vellard, 1948) (Anura, Ceratophryidae). South American Journal of Herpetology, 1(3), 202–209.
- Roçek, Z., Böttcher, R., & Wassersug, R. (2006). Gigantism in the tadpoles of the Neogene frog *Paleobatrachus*. *Paleobiology*, 32(4), 666–675.
- Rose, C. S. (2005). Integrating ecology and developmental biology to explain the timing of frog metamorphosis. *Trends in Ecology & Evolution*, 20(3), 129–135.
- Rot-Nikcevic, I., & Wassersug, R. J. (2003). Tissue sensitivity to thyroid hormone in athyroid *Xenopus laevis* larvae. *Development Growth Differentiation*, 2003(45), 321–325.
- Rot-Nikcevic, I., & Wassersug, R. J. (2004). Arrested development in Xenopus laevis tadpoles: How size constrains metamorphosis. The Journal of Experimental Biology, 207, 2133–2145.
- Ruibal, R. R., & Thomas, E. (1988). The obligate carnivorous larvae of the frog *Lepidobatrachus laevis* (Leptodactylidae). *Copeia*, 1988, 591–604.
- Schreiber, A. M., Liquan, C., & Brown, D. D. (2005). Remodeling of the intestine during metamorphosis of *Xenopus laevis*. Proceedings of the National Academy of Sciences, 102(10), 3720–3725.
- Smirnov, S. V., & Vassilieva, A. B. (2014). Thyroid hormones in the skeletogenesis and accessory sources of endogenous hormones in *Xenopus laevis* (Amphibia; Anura) ontogeny: Experimental evidence. *Doklady Biological Sciences*, 455(5), 136–138.
- Wassersug, R. J., & Heyer, W. R. (1988). A survey of internal oral features of Leptodactyloid larvae (Amphibia: Anura). Smithsonian Contributions to Zoology, 457, 1–96.
- Weber, G. M., Farrar, E. S., Tom, C. K. F., & Grau, E. G. (1994). Changes in whole-body thyroxine and triiodothyronine concentrations and total content during early development and metamorphosis of the toad *Bufo marinus*. *General and Comparative Endocrinology*, 94, 62–71.
- Zeng, C., Gomez-Mestre, I., & Wiens, J. J. (2014). Evolution of rapid development in spadefoot toad is unrelated to arid environments. *PLoS ONE*, 9(5). doi:10.1371/journal.pone.0096637.