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Vocal sac development and accelerated sexual maturity in the lesser swimming frog, *Pseudis minuta* (Anura, Hylidae)

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ABSTRACT

Sexual maturity involves the differentiation of the reproductive system, the maturation of germ cells, and the development of secondary sexual characteristics. Even though this topic has received much attention, little is known about the sequence of events that encompass reproductive maturation in anurans and what it could reveal about the developmental basis of life cycle evolution. The discovery of froglets of *Pseudis minuta* with incipient vocal sacs calling in breeding pools alongside several larger adult specimens with fully developed vocal sacs raised the question of the timing of sexual maturity in this species. Here we describe the sequence and timing of differentiation, development and maturation of the vocal sac apparatus and the testes in *P. minuta* (Anura, Hylidae), in order to establish a timeline of events leading to sexual maturity. Differentiation of the vocal sac apparatus begins at the final metamorphic stages, earlier than reported for other species, and the vocal sac acquires its final shape during the early postmetamorphic period. These modifications occur after gonadal differentiation, which begins early during the larval period and proceeds with a highly accelerated rate of development (e.g., secondary spermatids appear well before metamorphic climax), a situation reported previously for other anuran species only in the genus *Pseudis*. These results, together with a skeletochronological analysis showing that some calling specimens presented no lines of arrested growth, indicate acceleration in the timing of sexual maturity in *Pseudis*, and raise questions about the interdependence/decoupling during the development of the different components involved in reaching the adult stage.

1. Introduction

A life cycle can be defined as the complete ontogenetic series of developing and adult forms between a given developmental stage and the same stage in the next generation (Arthur, 2004), and implies a sequence of morphological changes undergone by an organism and the passage from one generation to the next through reproduction. In this context, we can identify a number of key events during a life cycle such as fertilization, hatching and sexual maturity that delimit the embryonic, juvenile and adult periods. In the case of complex or biphasic life cycles, a larval period succeeds the embryonic one and a new event, metamorphosis, can be recognized.

Most amphibians exhibit a complex life cycle, with aquatic egg and larval stages followed by a metamorphosis to terrestrial juvenile and then adult (Duellman and Trueb, 1986). In general, the life cycle of amphibians is regarded as a linear and rigid sequence of events and periods (usually presented in a circular form), but several cases show deviations from this generalization: in the urodele *Ambystoma mexicanum*, reproduction occurs in specimens that retain some larval features (Duméril, 1866), and historically this has been used as the main example for illustrating the term neoteny. In anurans, some individuals of *Sphaenorhynchus bromelicola* may reach sexual maturity (i.e., gonadal maturation represented by vitellogenic oocytes in females and spermatozoids in males, and secondary sexual characters such as the vocal sacs) while still retaining tails (Bokermann, 1974) but, unlike in urodeles, reproduction during the larval period has never been reported. Within the genus *Pseudis*, the species *P. paradoxa* and *P. platensis* exhibit a highly accelerated rate of germ cell development and it has been hypothesized that the juvenile stage is included within their prolonged delayed metamorphosis; therefore, sexual maturity is achieved as soon as the metamorphic period ends in an ontogeny that extends for three years at the most (Downie et al., 2009; Fabrezi et al., 2010).

In general, the juvenile stages have not received much attention despite the potential importance of knowing the ontogenetic trajectories of several organs and systems during this period. These stages mainly involve somatic growth and developmental processes associated with sexual maturity. In the absence of external morphological characters, adults can be recognized by the stage of gonad differentiation or arguably by their size (Quinzio et al., 2015), although developing secondary sexual characters could also be indicators of this period. It is unclear what role the early acquisition of sexual maturity, along with

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other parameters of the life cycle (e.g. the extension of the larval period, life span, growth rate, etc.), plays in the evolution of anurans.

Finding froglets of *Pseudis minuta* with incipient vocal sacs calling in breeding pools alongside several larger adult specimens with fully developed vocal sacs made us wonder if sexual maturity in this species is reached during or rapidly after metamorphosis (with the consequent early acquisition of secondary sexual characters and the ability to reproduce), and if this is concomitant with the unusual ontogenetic trajectory of *Pseudis* species and their unique life cycle. In fact, the timing of secondary sexual character differentiation (for instance, vocal sacs) in these species has not been determined yet. It is unclear when males and females are actually able to breed.

The possibility of a shift in the timing of sexual maturity in the genus *Pseudis* highlights the importance of studying gonadogenesis, age at sexual maturity and differentiation and development of secondary sexual characters as key features to understand the developmental basis of life cycle diversification. In this context, we here present a detailed description of the differentiation and development of the vocal sac apparatus in *P. minuta*, its adult structure, and the sequence of events related to testis differentiation and development. We present data on the age of juveniles and sexually mature specimens and a preliminary comparative analysis of calls of juvenile and adult specimens. These results allow us to describe the changes leading to the unusual life cycle of *Pseudis* species and to discuss the interdependence/uncoupling, during development, of the various components involved in the acquisition of sexual maturity.

2. Materials and methods

2.1. Specimens

We studied larval and postmetamorphic specimens of *P. minuta* (Tables 1 and 2). Specimens were euthanized in an aqueous solution of chloretone, fixed in 10% formalin and adults were preserved in 70% alcohol. All specimens were collected in temporary and semi-temporary ponds near the interception of El Pescado River and Street 31 (35°1′19.81″S, 57°51′10.11″W), La Plata department, province of Buenos Aires (Argentina), and in Punta Indio (35°35′39.3″S, 57°29′07.41″W), Punta Indio department, province of Buenos Aires (Argentina). Larval specimens, accessioned as lots, and adult specimens, accessioned with individual numbers, are deposited in the Herpetological Collection of the Instituto de Bio y Geociencias (IBIGEO)-CCT–CONICET Salta with the following collection numbers: larvae: IBIGEO-A 1581–1582; 1597–1600); postmetamorphic specimens: IBIGEO-A 1578–1580; 1583–1591; 1593–1596). Larval development was staged according to Fabrezi et al. (2009). We used this table because it recognizes, in *Pseudis platensis*, several more metamorphic stages than those described in the commonly used table of Gosner (1960). However, the approximate Gosner Stage is also given for comparative purposes (~followed by the stage numbers in Arabic numerals).

Table 1
Larval specimens of *Pseudis minuta* used to describe the vocal sac apparatus and testis development. Acronym with number of specimens in each lot (in parentheses). Roman numerals show the stages proposed by Fabrezi et al. (2009) and Arabic numerals those proposed by Gosner (1960). *n*, number of specimens used in the histological procedure.

Voucher	Stages	Sex	Testis histology *n* (Stages)	Sac dissection	Lower jaw histology
IBIGEO-A 1581 (6)	VIII-XI (~44–45)	3♂, 3♀	3 (VIII, IX, XI)	2♂, 2♀	none
IBIGEO-A 1582 (7)	X-XII (~45–46)	5♂, 2♀	4 (X, X, XI, XII)	5♂, 2♀	3♂ 2♀
IBIGEO-A 1597 (4)	VII-XI (~43–45)	2♂, 2♀	2 (X, XI)	2♂, 1♀	2♂
IBIGEO-A 1598 (7)	III-V (~35–42)	1♂, 2♀	1 (IV)	1♂, 2♀	none
IBIGEO-A 1599 (31)	I-III (~29–36)	12♂, 8♀	10 (I, II, III)	10♂, 6♀	none
IBIGEO-A 1600 (3)	XI-XII (~45–46)	3♂	1 (XII)	3♂	1♂

Table 2
Postmetamorphic specimens of *Pseudis minuta* used to describe age, vocal sac apparatus and testis morphologies in males, and the superficial throat musculature in females. Grey bar indicates the young specimen whose calling was recorded.

Voucher	Sex	SVL (in mm)	Gonad histology	Sac dissection	Lower jaw histology	LAGs
IBIGEO-A 1578	♀	40.90	no	no	yes	
IBIGEO-A 1580	♀	30.40	no	yes	no	
IBIGEO-A 1593	♀	39.92	no	yes	no	
IBIGEO-A 1594	♀	47.00	no	no	yes	
IBIGEO-A 1579	♂	29.10	no	no	no	1
IBIGEO-A 1583	♂	34.71	no	yes (half)	yes (half)	3
IBIGEO-A 1584	♂	27.06	yes	yes	no	2
IBIGEO-A 1585	♂	21.80	yes	no	Yes	0
IBIGEO-A 1586	♂	24.12	yes	yes	no	0
IBIGEO-A 1587	♂	26.94	yes	yes	no	0
IBIGEO-A 1588	♂	34.62	yes	yes	no	2
IBIGEO-A 1589	♂	33.50	no	yes	no	2
IBIGEO-A 1590	♂	36.58	no	yes (half)	yes (half)	4
IBIGEO-A 1591	♂	31.30	no	no	yes	4
IBIGEO-A 1595	♂	32.82	no	no	no	3
IBIGEO-A 1596	♂	25.36	yes	no	yes	0

2.2. Specimen preparation and histology

Data were obtained from the following sources: (i) Manual dissection of larval and postmetamorphic specimens to describe changes in gonads and superficial throat musculature. Description of the orientation of the muscle fibers was aided by staining them with iodine. (ii) Histological sections of gonads and lower jaw (vocal sac apparatus) made to study ontogenetic transformations in the transition from larva to adult. (iii) Counting the number of lines of arrested growth (LAGs) in transverse sections of phalangeal bones of toe IV in order to estimate the age of postmetamorphic specimens. For histological sections, lower jaws, gonads, and toe IV were separated from preserved specimens (lower jaw and toe IV were decalcified), dehydrated, embedded in paraffin, and sectioned at 6 μm for gonads and toes and 9 μm for lower jaws. Sections of phalangeal bones and gonads were stained with hematoxylin and eosin following the protocol of Martoja and Martoja-Pierson (1970), while lower jaws were stained with hematoxylin and eosin following the protocol of Martoja

and Martoja-Pierson (1970) and Masson's trichrome after Jones (2002). The analysis of LAGs was performed according to the technique detailed in Hemelaar (1986) by two independent viewers. Histological sections were given the same collection numbers as the vouchers and are deposited in the Herpetological Collection of the Instituto de Bio y Geociencias (IBIGEO). Descriptions, illustrations, and photographs were made with either a Nikon SMZ1000 stereo dissection microscope or a Nikon E200 microscope, both equipped with a digital camera. Snouth–vent length (SVL) in all specimens examined was measured with dial calipers (to 0.02 mm) and is expressed as mean ± SD.

2.3. Vocalizations

All vocalizations were obtained from the pond located near El Pescado River. We recorded one juvenile (SVL = 21.80 mm) on December 16th, 2011 using a Zoom H4n recorder (Zoom North America, Hauppauge, NY, USA) and a Sennheiser MKE 600 microphone (Sennheiser electronic, Wedemark, Germany) at 21:30 h, with 26.3 °C and 28.4 °C air and water temperature, respectively. To compare the juvenile call with those of adults, we used the calls of three adult *P. minuta* recorded on October 21st, 2011, at 22 h, at 20 °C and 23 °C air and water temperature, respectively. The vocalizations were recorded at 2–3 m distance from each male adult with a digital recorder (Roland Edirol R-09HR; Roland Corp., Osaka, Japan) equipped with an external microphone (ATR-55; Audio-Technica, Machida, Japan). All digital recordings were sampled at 44.1 kHz sampling rate, with a resolution of 16 bit, saved in uncompressed wave files. Vocalization analyses were performed with the software Adobe Audition v1.5 (FFT of 1024 points, 44.1 kHz sampling rate, 16-bit precision). The terminology used for advertisement call comparison follows Heyer et al. (1990) and Zank et al. (2008).

2.4. Description and terminology

We first describe the morphology of the vocal sac apparatus in adult males and then its differentiation and development, in order to show the structure of the apparatus and then the events and the sequence that take place to achieve the final shape. We follow the terminology of Liu (1935), Inger (1956) and Noble (1931) for the external morphology of the vocal sac apparatus, and Tyler (1971) for the superficial mandibular musculature and vocal sac apertures.

3. Results

3.1. Vocal sac apparatus in adults

The adult vocal sac apparatus in *P. minuta* (Figs. 1 and 2A) consists of: (1) a pair of vocal sacs, subgular in position with medial adhesions that prevent communication between both portions, (2) an internal layer of superficial modified mandibular muscles (the m. intermandibularis and the m. interhyoideus), (3) an external layer of modified gular skin, and (4) the vocal sac apertures, which are large slits (40% of the length of the mandible) located approximately midway along the length of the mandible. Externally, each sac protruded very close to the mandibles on the ventral surface of the throat through a highly folded, longitudinal cleft in the skin. The cleft extended from a point at the nostril level to the posterior end of the tympanum (Fig. 1). In the deflated position, the vocal sacs were visible beneath the clefts as pleats of wrinkled and well pigmented skin (Fig. 1A).

The histological analyses of the vocal sac apparatus revealed a modified gular skin involving both the epidermis and the dermis (Fig. 3A–D). In sagittal view, the anterior and posterior limits of the vocal sacs were evidenced by histomorphological changes in the skin (Fig. 3A). The epidermis was a multilayered epithelium in which 4–5 cell layers of basal, germinative and granular cells were present (Fig. 3B). Together with the dermis, the epidermis of the apparatus showed a strong plication in the deflated position (Fig. 3C and D). The thickness of the dermis showed a great reduction in relation to the rest of the gular skin; the stratum spongiosum was much reduced and only numerous and regularly dispersed melanocytes and a small number of mucous glands could be observed in this layer (Fig. 3C and D). The collagen lamellae of the stratum compactum were, like the epidermis, strongly plicated. All of the skin involved in the vocal sacs formed a pouch of folded skin (Fig. 3A).

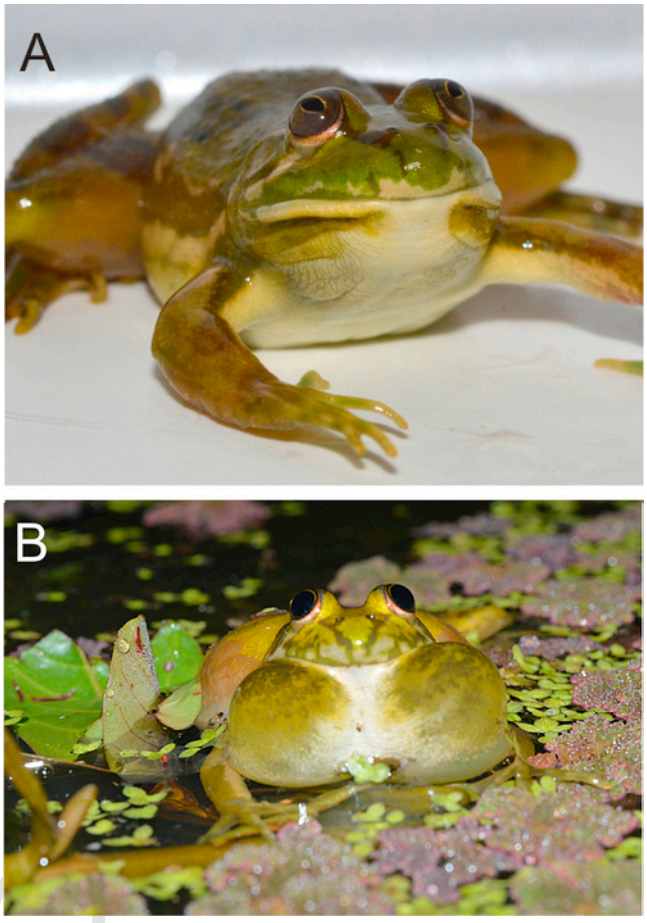

Fig. 1. Adult male of *Pseudis minuta* with (A) deflated and (B) inflated vocal sacs.

The m. intermandibularis arose from the lateral surface of the mandible and extended from the m. submentalis anteriorly to three quarters of the lower jaw posteriorly (Fig. 4A). It was not differentiated (i.e., it lacked supplementary elements). The anterior half of the fibers of the muscle met at a medial raphe; the posterior half of the muscle radiated from a medial aponeurosis which was of a variable shape (Fig. 4A). This aponeurosis extended posteriorly into the m. interhyoideus. The m. interhyoideus extended anteriorly to the post-articular extremities of the mandibles and lay upon the sternal musculature; it was connected to the m. episternohumeralis by a broad sheet of collagenous connective tissue. Fibers of the m. interhyoideus inserted along a medial raphe extending from the terminus of the intermandibular aponeurosis. The m. interhyoideus appeared hypertrophied with the presence of antero- and posterolateral, globular-

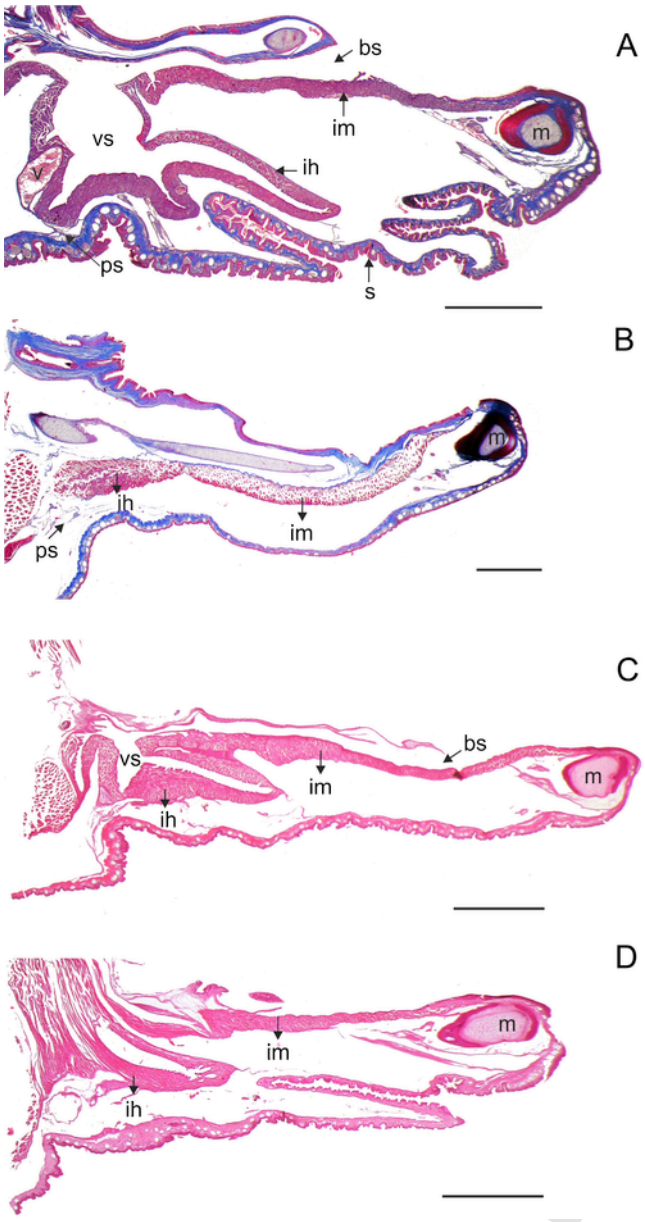

Fig. 2. Comparison of sagittal sections of the lower jaw in postmetamorphic specimens of *Pseudis minuta*. (A) Vocal sac apparatus in an adult male. Note the increased development of the m. interhyoideus and its high degree of vascularization, and the large folding of the underlying skin. (B) Adult female without the hypermorphic development that characterizes males. (C and D) Serial cross-sections of the vocal sac apparatus in the recently metamorphosed male that was calling in the pond. Note the incipient development of those components which form the apparatus, the m. (C) and the throat skin (D). Scale bars = 1 mm. *Abbreviations*: bs, buccal slit; m, mandible; ih, m. interhyoideus; im, m. intermandibularis; ps, postmandibular septum; s, skin; vs, lumen of the vocal sac.

shaped, highly vascularized bulges in close contact with the inner layer of the gular skin (Fig. 4A). Anteriorly, these structures covered the posterolateral end of the m. intermandibularis, while posteriorly they rested on the sternal musculature.

Sagittal sections of the lower jaw showed the highly folded and pleated configuration of these bulges that extended almost parallel to the skin folds (Fig. 2A). The internal surface of the hypertrophied muscle, facing the lumen of the vocal sac, was composed of a single-layered columnar epithelium including a few ciliated cells. The epithelial layer contained many folds (Fig. 2A). Fibers located midway along the muscle attached directly to the underlying skin at different points (Fig. 2A). The postmandibular septum, composed of a transparent, membranous tissue, was of moderate length and appeared attached to the ventral surface of the posterior end of the m. interhyoideus, which extended slightly into the pectoral lymph sac (Fig. 2A).

There is no vocal sac in female *P. minuta* (Figs. 2B and 4B). The female gular skin had a typical adult anuran skin configuration (Figs. 2C and 3E). It resembled the gular skin of the juvenile males (see Section 3.2; Fig. 3F), although in adult females there was a slight increase in the thickness of the different layers of the integument. The m. interhyoideus did not appear hypertrophied, in fact it looked quite similar to the m. intermandibularis and it was less vascularized than in males (Figs. 2B and 4B). The postmandibular septum also showed sexual dimorphism; in females the septum is attached at the posterior end of the m. interhyoideus.

3.2. Development of the vocal sac apparatus

From early larval to early metamorphic stages (Stages I–VII of Fabrezi et al., 2009; ~Stages 26–43 of Gosner, 1960) *P. minuta* exhibited the typical larval skin configuration with a two-layered epidermis and a dermis in which only the stratum compactum was present (Fabrezi et al., 2010). The m. submentalis was absent. The m. intermandibularis was a small and thin, ventrally curved, paired muscle with transversely extended fibers (Fig. 4C). This muscle arose from the ventral surface of Meckel's cartilage; the anterior fibers were parallel, while the posterior ones spread postero-medially in a curved manner. All fibers attached to those of the opposite side through a medial raphe (Fig. 4C). The m. interhyoideus was a paired, ribbon-like, transverse muscle with parallel fibers arising from the ventral surface of the ceratohyal. All fibers met at a medial ventral raphe. This condition of the superficial mandibular muscles remained without changes through late metamorphic stages.

By Stage VIII (~44), the m. submentalis appeared as a very small, araphic, medial muscle (Fig. 4D). It consisted of a small number of parallel fibers with a slightly angled anterior part near the symphysis of the lower jaw and curved posterior fibers. There was no contact with the m. intermandibularis. The m. intermandibularis changed in form, expanding its insertion on Meckel's cartilage, and the fiber direction changed from U-shaped to transverse with the origin slightly anterior to the medial contact. This site continued as a medial raphe, although it was more developed than in previous stages. The m. interhyoideus showed no changes in the general shape and arrangement of the fibers.

Later, in Stage X (~45), the skin presented changes related to the metamorphic transformations. The epidermis possessed three layers of cuboidal cells. Scattered serous and mucous glands were visible in the developing stratum spongiosum of the dermis. An increase in the size of the m. submentalis was evident; the posterior fibers curved and the characteristic triangular shape of the muscle was achieved. At this stage the two sexes diverged, with an increased development in males of the m. intermandibularis and the m. interhyoideus as well as a difference in the shape of the intermandibular aponeurosis (Fig. 4E–J). In males, both muscles had grown considerably and, as a consequence, the posterior fibers of the m. intermandibularis were located near the anterior edge of the m. interhyoideus (Fig. 4E). All fibers were transversely disposed and the medial raphe had developed into a medial aponeurosis (Fig. 4E). The m. interhyoideus curved slightly in an anterior direction. The medial raphe was wider anteriorly and it was closely related to the aponeurosis of the m. intermandibularis. Posteriorly, the raphe showed no change (Fig. 4E). By contrast, in females, the medial aponeurosis was still absent and the m. interhy-

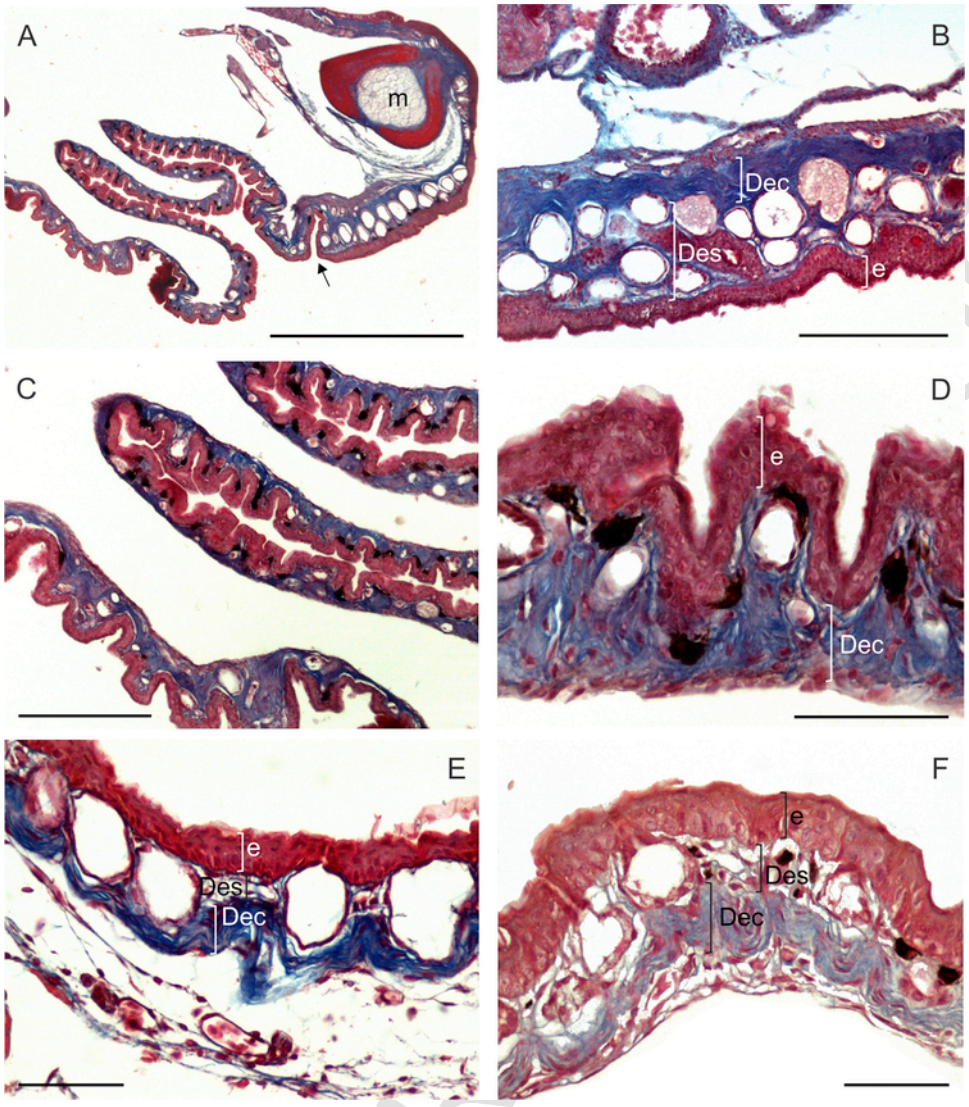

Fig. 3. Throat skin in postmetamorphic specimens of *Pseudis minuta*. (A–D) Adult male. The skin of the vocal sac apparatus appears folded and pleated (A, C, D), while the skin beyond the apparatus (B) appears with a typical adult configuration. The arrow in (A) indicates the anterior limit of the vocal sac apparatus. (C) Detail of the folded skin. (D) Detail of the modified skin of the apparatus. (E) Throat skin of the recently metamorphosed male. (F) Throat skin in an adult female. Scale bars = 1 mm in A and B, 0.25 mm in C, and 0.05 mm in D, E, and F. *Abbreviations*: Dec, stratum compactum of the dermis; Des, stratum spongiosum of the dermis; e, epidermis; m, mandible.

oideus showed no sign of size increase; the gap between this muscle and the intermandibularis was large (Fig. 4H).

In the next stages [XI–XII (~45–46)], toward the end of metamorphosis, in males the ventral skin began to show the first signs of folding. The m. submentalis and the m. intermandibularis increased in size and came into close contact with each other. The curvature of the m. interhyoideus increased (Fig. 4F). The thickness of this muscle varied: it was thicker in the lateral portion and thinner close to the medial raphe. In females this condition remained up to adult stages, while in males the curvature of the lateral edge of the muscle became more conspicuous (Fig. 4I and J). At the end of metamorphosis, the m. interhyoideus in males had increased in size and appeared slightly expanded laterally in close relationship with vocal sac differentiation (Fig. 4G). During the whole larval period, vocal sac apertures were absent and there was no connection between the buccal cavity and the ventral muscles.

In males, just after metamorphosis, the gular skin presented an incipient folding in the region where the adult vocal sacs protrude (Fig. 2D), but there were no signs of dark pigmentation. Vocal sac apertures differentiated next to the mandibles (Fig. 2C), at both sides of the tongue, already as large slits. In sagittal sections, the gular skin showed an epidermis with five or six layers of stratified basal, germinative, and granular cells (Fig. 3F). In the dermis, the stratum spongiosum contained melanocytes, which were located just beneath the basal layer of the epidermis, as well as numerous serous and mucous glands (Fig. 3F). In the stratum compactum of the dermis, undulated lamellae of collagen were present. The m. interhyoideus appeared pleated and slightly folded, although to a much lesser extent and with fewer connections with the gular skin compared with the condition in adults (Fig. 2D).

3.3. Testicular differentiation

By Stage I (~Stage 29 of Gosner) testes appeared as cylindrical cords with no external sign of differentiation beyond the absence of the lobulation that characterizes ovarian differentiation. The anlage of

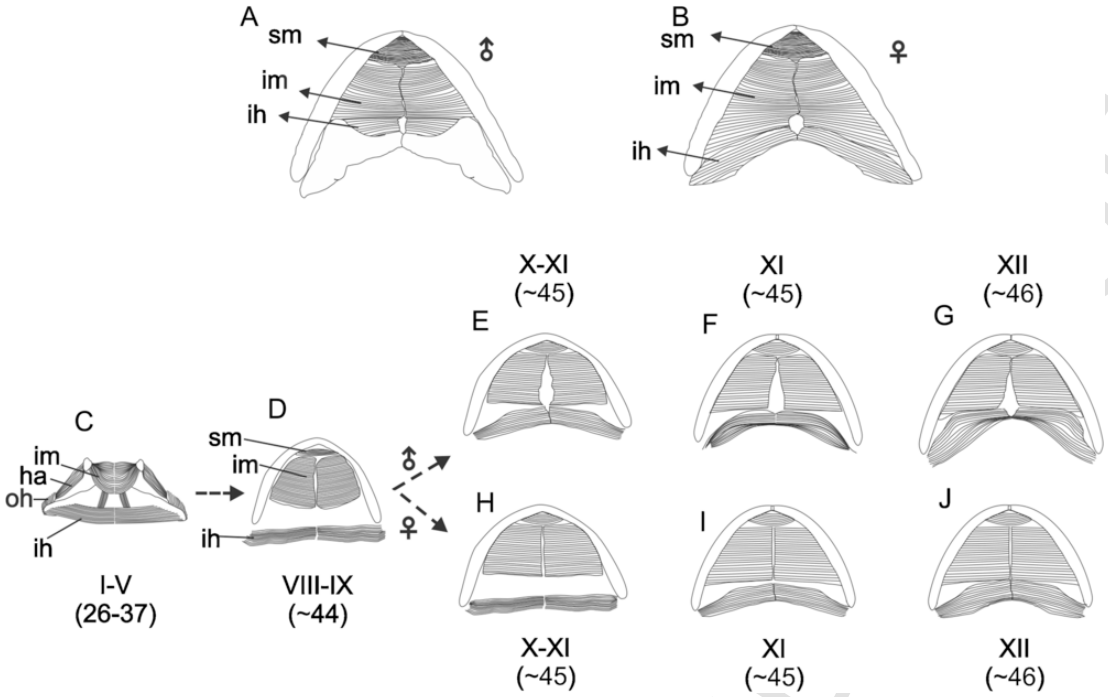

Fig. 4. External throat musculature in *Pseudis minuta*. (A) Adult male configuration. (B) Adult female configuration. (C–J) Development of the external throat musculature in both sexes. Each drawing also gives the developmental larval stage following Fabrezi et al. (2009) and Gosner (1960) (with Roman and Arabic numerals, respectively). Initial development follows a similar pattern between both sexes (C and D) up to stage X (~45) when sexual divergence occurs. Not scaled. *Abbreviations*: ha, m. hyoangularis; ih, m. interhyoideus; im, m. intermandibularis; oh, m. orbitohyoideus; sm, m. submentalis.

the fat bodies developed as fingerlike projections from the medial anterior end of both testes, with a whitish-translucent color. Histologically, each testis appeared as a massive structure with numerous medullary cells and germ cells in mitotic division. In the medulla, voluminous primary spermatogonia were evenly distributed and exhibited prominent nuclei with a single eccentric nucleolus. Flattened, crescent-shaped, darkly stained somatic cells were also present. Later (~Stage 30 of Gosner), though the testes remained without external changes (Fig. 5A), primary spermatogonia divided mitotically and produced clusters of smaller cells, the secondary spermatogonia, which remained together. Germ cells started to aggregate and the formation of a lumen in the testis cords indicated the onset of seminiferous tubule differentiation (Fig. 5D).

At Stage III (~Stages 35–36) the shortening and thickening of the testes was evident; the development and differentiation of the proximal part proceeded while the distal part degenerated, and there were only vestiges of it left (Fig. 5B). Differences in size between the left and the right testis became conspicuous. Pigmentation was distributed along the interstitial spaces so the differentiated seminiferous tubules could be visually identified inside the testes. At this stage the testes presented primary and secondary spermatogonia and spermatocytes in different phases of the first meiotic division with different degrees of condensation of the chromatin (Fig. 5E). By the beginning of Stage IV (~Stages 37–38) the testes acquired their final ovoid shape and fat bodies became yellowish (Fig. 5C). Testicular histology showed a well-developed tunica albuginea enveloping the whole testis. The testes appeared organized in abundant seminiferous tubules with several cysts of germ cells at different stages of differentiation. Within each cyst, germ cells were at approximately the same stage. Several cysts contained spermatids with different degrees of differentiation, from rounded spermatids I to elongated spermatids II that appeared strongly associated with Sertoli cells (Fig. 5F). These latter cells were easily identified by their darkly stained nuclei and their location near the locular periphery. By this stage some cysts began to show a large central vacuole and elongated spermatids appeared organized into bundles sustained by the Sertoli cells, signaling the beginning of spermiogenesis (i.e., the transformation from spermatid to spermatozoid).

In later stages, during metamorphosis, the testes continued to increase in length (Fig. 5G and H). The pigmentation was variable, from slightly to completely pigmented. Differentiation of elongated spermatids continued, forming evident bundles with their heads oriented toward Sertoli cell nuclei, usually located at the base of the tubule (Fig. 5J and K). Before the end of metamorphosis, the walls of several cysts of spermatids were already dissociated and these germ cells appeared to be released into the lumen of the seminiferous tubules (Fig. 5J). Spermatogonia and cysts of spermatocytes were also observed during these stages. Within the interstitial compartment, the rete testis differentiated from a cluster of medullar cells and Leydig cells increased in number and nuclear size (Fig. 5J). The condition observed during metamorphosis, in which many secondary spermatids appeared in advanced stages of spermiogenesis, continued during the juvenile stages (Fig. 5I and L).

Adult testes were paired, ovoid structures, with a massive presence of pigmented cells. Their size was almost four times that of the testes in metamorphic specimens. Seminiferous tubules exhibited an average diameter of 295 ± 41.05 μm (N = 10), and contained cells in all germinal stages. The interstitial compartment contained steroidogenic Leydig cells, connective tissue, and blood vessels.

3.4. Postmetamorphic growth and ageing

The analysis of gonadal differentiation and maturation together with the skeletochronological analysis of juvenile and adult specimens provided indications of the species' life span and age when sexual maturity is attained. The phalangeal cross-sections in four postmetamor-

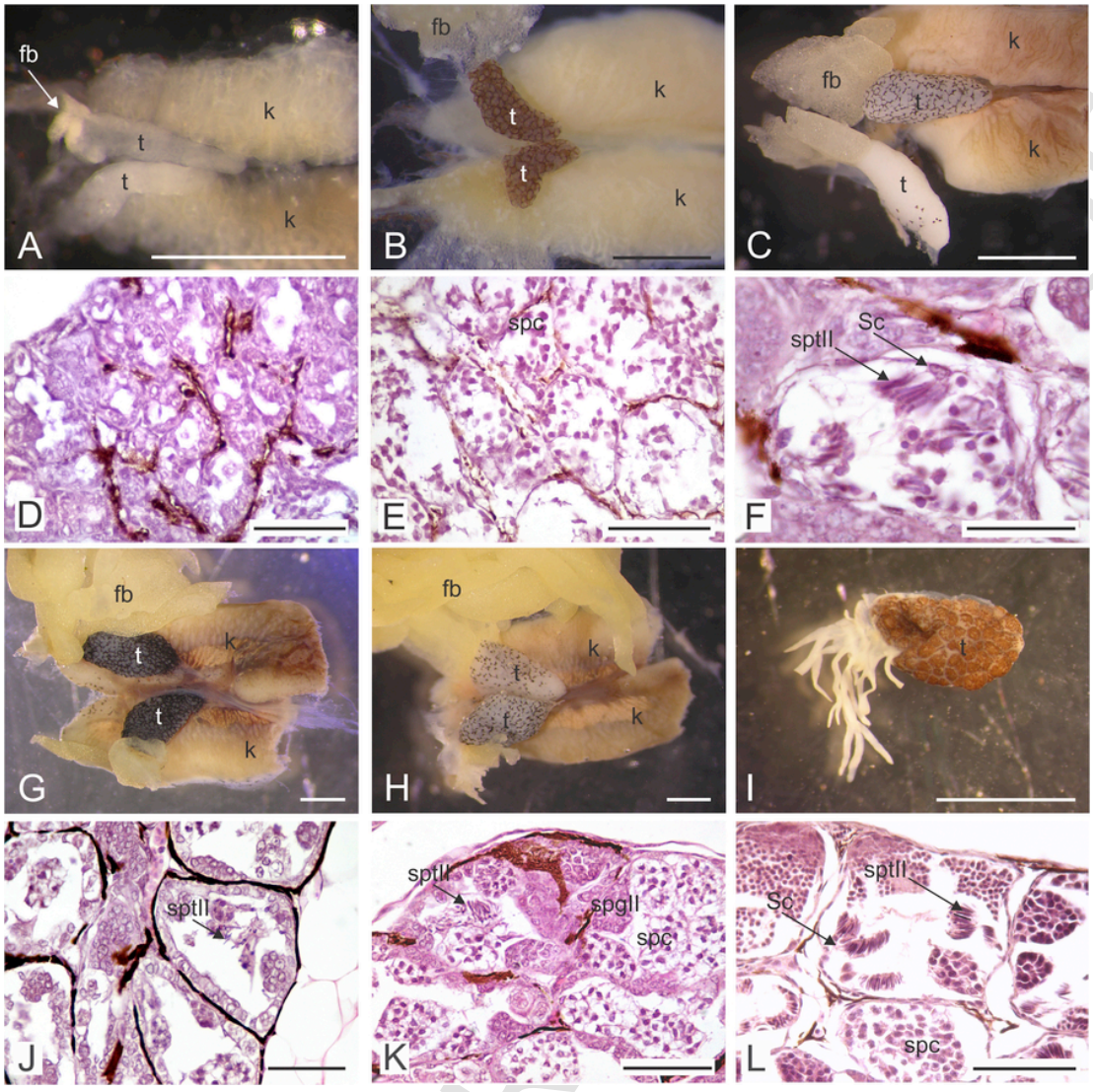

Fig. 5. Sequence of events during testes differentiation in *Pseudis minuta*. (A and D) Stage I (~30). Externally, testes show no sign of differentiation (A). Formation of seminiferous tubules begins through the differentiation of the lumen of each tubule (D). (B and E) Stage III (~36). Seminiferous tubules can be identified as small, rounded structures distributed all along the testes (B). Spermatocytes differentiate and appear in large numbers (E). (C and F) Stage IV (~37–38). Testes appear ovoid in shape with an irregular pattern of pigmentation (C). Secondary spermatids are already differentiated and appear as bundles of cells related to a single Sertoli cell (F). (G and J) Stage X (~45). Testes get thicker although they are smaller than in postmetamorphic stages (G). The number of secondary spermatids increases and the walls of the cysts that contain them can appear dissociated (J). (H–K) Stage XI (~45). Seminiferous tubules have several cysts with spermatogonia, spermatocytes and spermatids (K). (I–L). Testes in a recently metamorphosed male that was calling in the pond. The testes are larger than in the previous stages and the numerous seminiferous tubules can be identified (I). Seminiferous tubules present germ cells in different stages of differentiation: spermatogonia, spermatocytes, and numerous spermatids (L). Scale bars = 1 mm in A–C, G–H; 0.05 mm in D, F, J; and 0.1 mm in E, K, L. *Abbreviations*: fb, fat bodies; k, kidney; t, testis; spc, spermatocytes; Sc, Sértoli cell; spgII, secondary spermatogonia; sptII, secondary spermatids.

phic specimens with an average SVL of 24.55 mm were composed of a single layer of avascular and parallel-fibered bone encircling a wide medullary cavity. In adults, the results revealed: 1 LAG (one male with SVL = 29.1 mm); 2 LAGs (three males with SVL = 27.06 mm, 33.5 mm, and 34.6 mm); 3 LAGs (two males with 32.82 mm and 34.71 mm); and 4 LAGs (two males with SVL = 31.3 mm and 36.6 mm). Larval specimens at the end of metamorphosis [i.e., stages X–XII (~ 45–46)] had an average SVL of 17.6 ± 0.49 mm, juveniles had an average SVL of 24.55 ± 2.16 mm, while in adult males it was 32.46 ± 3.15 mm. This means that postmetamorphic growth involved an increase of 1.84 times the metamorphic size. These data suggest that: (i) there is postmetamorphic growth; (ii) the juvenile stage lasts a few months, up to the first latency period when sexual maturity is attained; and (iii) sexually mature specimens can live at least 4 years after metamorphosis.

3.5. Vocalizations

The vocalizations of adult *P. minuta* were already described by Zank et al. (2008); we aim here to report the call of a juvenile, with a brief comparison to adult calls. The adult advertisement call from the study site showed great variations (Fig. 6). With the few samples analyzed (26 calls of three adults) we found that the advertisement call could be a single call or repetitions from 3 to 13 calls with intervals of 0.51 ± 0.13 s (range: 0.37–0.94 s) between them. The call duration

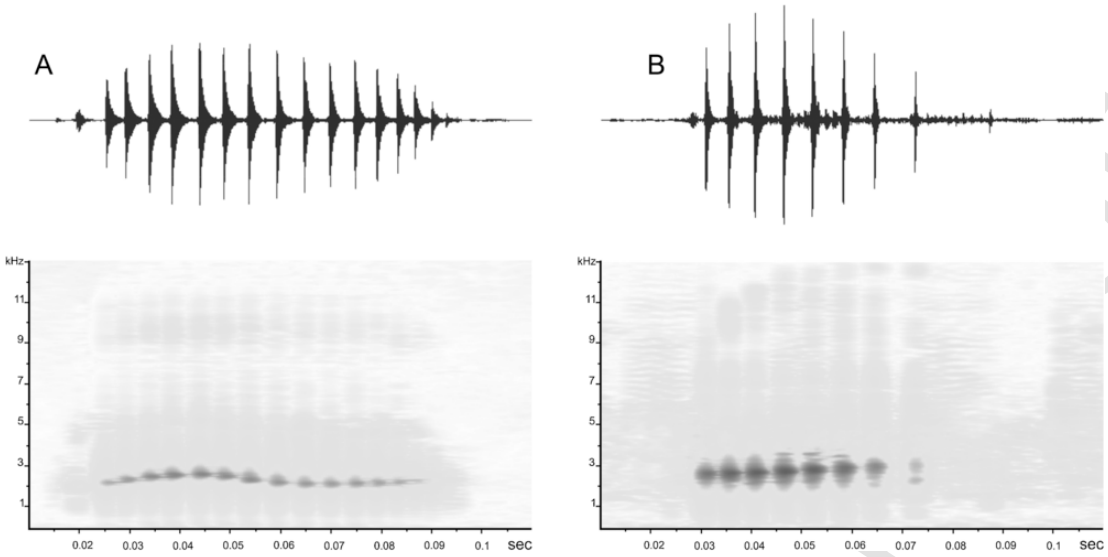

Fig. 6. Comparison of calling between (A) adults and (B) young specimens. Both calls differ in the number of pulses, which is lower in the young, and dominant frequencies of pulses. Waveforms are shown on top, and in the sound spectrograms on the bottom, time is presented in seconds (sec).

was 0.12 ± 0.03 s (range: 0.07–0.16 s). Each call was composed of multiple pulses that showed variation in number between and within individuals, ranging from 8 to 19 pulses (mean: 13.62 ± 3.57). In those cases with several repeated calls, the first and last calls had fewer pulses than the middle calls. Additionally, each pulse had a different dominant frequency (Fig. 6A). In the first half of the call the dominant frequency of each pulse can be described as a concave curve, and the second half of the pulses as a convex curve. The mean dominant frequency of calls (taken as the mean dominant frequency of each pulse) was 2329.66 ± 443.9 kHz, with a maximum at 3120.80 kHz and a minimum at 813.8 kHz. In addition to advertisement calls, adults performed aggressive calls as well (Zank et al., 2008), but we will not consider them here.

The juvenile analyzed (six calls of one exemplar) vocalized in repetitions of three calls composed of 7–9 pulses in each call (Fig. 6B). Duration of each call was 0.094 ± 0.018 s (range: 0.069–0.115 s), with intervals of 0.737 ± 0.242 s (range: 0.544–1.06 s) between them. The mean dominant frequency of the calls (taken as the mean dominant frequency of each pulse) was 2629.14 ± 469.35 kHz, with a maximum at 3079.1 kHz and a minimum at 1226.0 kHz. The limited sample did not allow us to perform a statistical analysis, but we could observe differences in modulation. The dominant frequency of each pulse of the juvenile call presented a slight linear increase (Fig. 6B) from the first to the penultimate pulse; in the last pulse the frequency dropped sharply. Aggressive calls were not detected in juveniles.

4. Discussion

In adult male anurans the major secondary sexual characteristics, such as the vocal sacs and the nuptial pads, are indicative of testicular androgen formation and reproductive activity (Emerson, 2000). In general, secondary sexual characters have been examined from three main perspectives: (i) the paradigm of sexual selection, with traits considered as selected or adaptive features of adult stages (e.g., Hedrick and Temeles, 1989; Zank et al., 2008), (ii) the physiological processes controlling sexually dimorphic morphologies, through the differential effects of steroid sex hormones (reviewed in Emerson, 2000), or (iii) as taxonomic characters used in phylogenetic reconstruction (e.g., Liu, 1935; Liem, 1970; Tyler 1971, 1972, 1974, 1985; Hayes and Kremples, 1986; Tyler and Duellman, 1995; Lambertz et al., 2014). There are very few studies that address the timing of differentiation and development of secondary sexual characters as developmental evidence of sexual maturity, and especially their synchrony/asynchrony with gonadal maturation (Olmstead et al., 2009), a necessary event in reaching the adult stage.

4.1. Vocal apparatus

In *P. minuta,* and at least in those other species that present paired and external vocal sacs (Liu, 1935; Tyler, 1971), the vocal sac apparatus is a complex structure which involves anatomical components found in both sexes, such as the gular skin and the superficial throat musculature, and new structures, such as the vocal sac *per se* and the vocal apertures. Several authors (Inger, 1956; Inger and Greenberg, 1956; Tyler 1971) described the development of the superficial ventral muscles in the context of vocal sac variation, but comprehensive ontogenetic studies dealing with the development of the whole apparatus have not been undertaken.

The modifications of the gular skin from the larval to the adult configuration follow the pattern described for the ventral body skin in *P. platensis*, in which most changes occur during metamorphosis (Fabrezi et al., 2010). However, it seems that these changes occur with a different timing in males than in females of *P. minuta*, at least in the gular skin, because at Stage X (~ 45) males present a more developed skin with thicker components. Even if the characteristic skin of the vocal sac apparatus, with folds, pleats, and dermal modifications, is not discernible until the end of the metamorphosis, it is evident that the onset of the dimorphism in the skin configuration begins at the late metamorphic stages.

Studies on the metamorphosis of the superficial throat muscles show that all species studied so far exhibit a similar sequence and timing of development, with increased rates of development and growth at the final stages (Sedra, 1950; De Jongh, 1968; Tyler, 1971). Our results for *P. minuta* are consistent, in general, with these descriptions. However, none of them mentions the possibility of sexual differences during the process. Interestingly, as in the skin, in *P. minuta* the m. intermandibularis and the m. interhyoideus exhibit a similar pattern of development in both sexes up to Stage IX (~Stage 44), nearly the end of metamorphosis, when several male-specific changes occur in their size and shape (Fig. 4): (i) the differentiation of a medial aponeurosis

all along the m. intermandibularis and the m. interhyoideus, which continues differentiating up to the juvenile stage, (ii) the predisplacement of the fusion of the m. intermandibularis with the anterior border of the m. interhyoideus, which in females and in other species (Tyler, 1971) is postmetamorphic, and (iii) an increase in the volume of the m. interhyoideus. Beyond the question of whether all these changes are prerequisites for muscular hypertrophy, they mark the onset of the process of maturation of the male vocal apparatus, which continues its development immediately after metamorphosis.

The structural modifications of the m. interhyoideus are directly related to the onset of the vocal sac differentiation in *P. minuta*. A defined vocal sac differentiates concomitantly with muscle hypertrophy and folding. In turn, vocal sac apertures differentiate as the last step. There is not much information among anurans about the development of the vocal sac apparatus to compare with these data. In *Sclerophrys regularis*, Inger (1956) and Inger and Greenberg (1956) described a developmental pattern similar to that of *P. minuta* with respect to the relationship between the vocal sac and the m. interhyoideus, but they found a different pattern in *Ptychadena porosissima*, probably because of the extreme attachment of this muscle to the gular pouch. Nonetheless, the onset of the differentiation of the vocal apparatus in these species occurs at postmetamorphic stages and after a period of growth, but with a similar sequence, also with the formation of the apertures as the last event. Other secondary sexual characters, such as the nuptial pads or the larynx, also begin to differentiate several months after metamorphosis (Sassoon and Kelley, 1986; Kelley, 1996; Olmstead et al., 2009). Therefore, in *P. minuta* the differentiation and development of the vocal sac apparatus is accelerated; by the end of metamorphosis the main traits have begun their changes and they acquire their final shape soon after metamorphosis.

The sequence of events related to the differentiation and development of the vocal sac apparatus evidence that: (i) both sexes begin to diverge during metamorphosis and (ii) all components follow their own individual sequence and timing to converge in the adult structure.

4.2. Testis differentiation

P. minuta exhibits a type of gonadal sex differentiation classified as "differentiated" (Gramapurohit et al., 2000), which means that indifferent gonads directly differentiate into an ovary or a testis. Goldberg (2015) distinguished three types of developmental rates of testis and germ cells relative to somatic development: basic, retarded, and accelerated. In the basic and retarded types, the differentiation of the seminiferous tubules of the testes occurs during or after metamorphosis, respectively. *P. minuta*, by contrast, displays the accelerated type: spermatogonia differentiate in earlier tadpoles (during limb bud appearance), and testicular development progresses at a rapid rate, with seminiferous tubules present long before metamorphosis. The accelerated pattern has also been recorded for *P. paradoxa* but, interestingly, *P. platensis* presents the basic type (Downie et al., 2009; Fabrezi et al., 2010).

In *P. minuta* the first (histological) sign of testicular differentiation takes place before that of ovarian differentiation, and, although it exhibits an accelerated pattern as do other species, the very early differentiation of seminiferous tubules has only been described, among anurans, in *P. paradoxa* (Downie et al., 2009). Furthermore, both species are the only described species, with the exception of occasional cases of *Sphaenorhynchus bromelicola* (Bokermann, 1974) or athyroid tadpoles of *Xenopus laevis* (Rot-Nikcevic and Wassersug, 2004), in which spermatids are in advanced stages of spermiogenesis long before metamorphosis. In fact, in *P. minuta*, secondary spermatids appear already embedded in a single Sertoli cell and the cysts are broken, indicating the formation of the spermatid flagellum (Pudney, 1995). In parallel, females exhibit diplotene oocytes, ready for the vitellogenic phase, in premetamorphic tadpoles (Goldberg, pers. obs.).

The spermiogenic process has been described in several anuran species, but always at postmetamorphic stages (e.g., Iwasawa and Kobayashi, 1976; Gramapurohit et al., 2000; Ogielska and Bartmańska, 2009). Its early onset in *P. minuta* and *P. paradoxa* represents an outstanding and unique case among anurans. In postmetamorphic specimens of *P. minuta* with no LAGs, spermiogenesis proceeds and Leydig cells increase in number and size with respect to metamorphic stages. These cells are responsible for the release of the androgens that regulate the final stages of spermiogenesis and spermiation (i.e, the process of sperm release), and secondary sexual characters as well (Propper, 2011). In general, spermiogenesis in anurans takes 7–45 days, depending on the species and/or the environmental conditions (Kalt, 1976; Rastogi et al., 1976; Neyrand de Leffemberg and Exbrayat, 1995), while spermiation occurs just before or during amplexus (Pudney, 1995). All this reinforces the notion of a fast acquisition of germ cell maturity within the genus *Pseudis*, either at the end of the larval stage or during the first growth period after metamorphosis.

4.3. Timing of sexual maturity in P. minuta

Sexual maturity represents a key event during a life cycle that separates the juvenile from the adult stage. When data on the timing and sequence of gonadal development and the differentiation of secondary sexual characters are lacking, there is a high risk of confusing an immature stage (juvenile) with the final stage (adult), or of not being able to distinguish a juvenile male from an adult female without looking at the gonads. In this context, *Pseudis* represents a clear example that illustrates the value of such information.

In *P. minuta* the differentiation of the gonads occurs quite early during larval stages and spermiogenesis proceeds up to an advanced point during the metamorphic stages, while the differentiation of the vocal sac apparatus begins before the end of metamorphosis (Fig. 7). All these data suggest that male sexual maturity in *P. minuta* can occur as soon as metamorphosis is complete, and, in fact, the young specimens reported here calling in ponds presented no LAGs yet their gonads were in advanced stages of spermiogenesis. However, even when young, male specimens differed from females because they already presented an incipient folding in the gular skin and in the m. interhyoideus (necessary for their calls), they lacked the dark pigmentation characteristic of reproductive males, which is also an indicator of androgen level (Sever and Staub, 2011), and they still had to grow to acquire adult size.

Fig. 7 depicts the sequence and timing of differentiation, development and maturation of the testes and vocal sac apparatus in *P. minuta* in order to describe a timeline of events leading to male sexual maturity in this species. When the ontogenetic trajectory of *P. minuta* is compared to that of other species of the genus (Downie et al., 2009; Fabrezi et al., 2010), it is evident that the accelerated acquisition of sexual maturity (i.e., maturation of gonads and secondary sexual characters) in *Pseudis* is related to a (very) early onset of spermiogenesis. Thus, *P. minuta* has a very short juvenile stage as evidenced by advanced spermiogenesis, as well as earlier vocal sac differentiation and function. The advanced gonadal differentiation and the early differentiation of the vocal sac apparatus in the genus *Pseudis* raise the question of the implications of an early sexual maturity in life cycle evolution. It is interesting that a species with a long larval period such as *P. platensis* (six months; Fabrezi et al., 2009) and a species such as *P. minuta* (two months; Agostini and Barrasso, pers. obs.), share a short

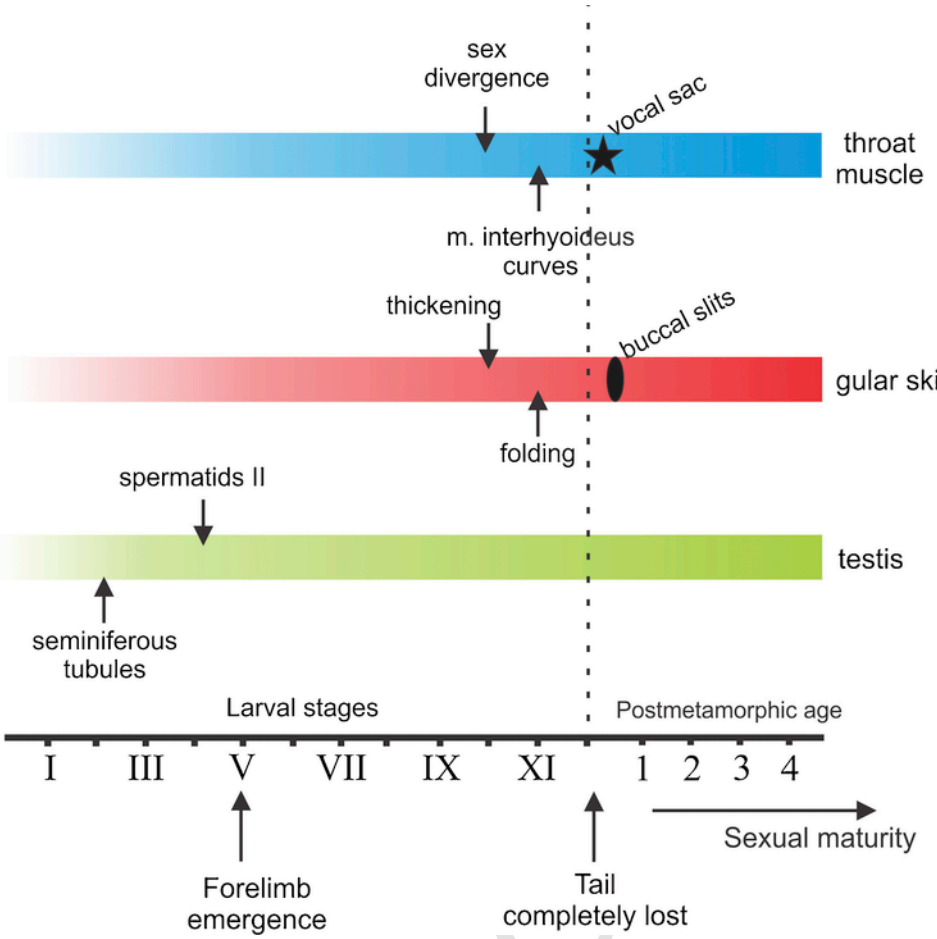

Fig. 7. Timeline of developmental changes in the vocal sac apparatus and gonadal differentiation during the larval and postmetamorphic periods in *Pseudis minuta*.

life cycle and an early sexual maturity but differ in their growth patterns: the former reaches its maximum size at the end of metamorphosis, while the latter exhibits postmetamorphic growth. This fact indicates that gonadal maturation is independent of somatic growth. Also, our observations prove that the development of primary and secondary sexual characteristics is independent of metamorphosis, because they may develop either before or after it. This dissociability of the three 'fundamental processes' of ontogeny (growth, maturation, and development according to Needham, 1933) constitutes the developmental pathway to evolutionary change.

In direct-developing species it has been shown that several larval structures (e.g., dermal folds, vent tube) appear predisplaced to the embryonic period, implying that even when the larval period is eliminated some typical larval traits can remain unchanged (Goldberg et al., 2012). In *Pseudis* we found evidence of how, in a short life cycle, the juvenile period can be fully or partially eliminated as a consequence of an early sexual maturity. Taken together, these findings underscore that there are different and complex ways in which anuran life cycles can evolve.

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