Heterochrony During Skeletal Development of *Pseudis platensis* (Anura, Hylidae) and the Early Offset of Skeleton Development and Growth

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ABSTRACT The aquatic frog *Pseudis platensis* has a giant tadpole, long developmental time, and dissociated metamorphic events that include later offset of larval somatic morphologies. Moreover, when the tadpole metamorphoses, the young frog is nearly the size of an adult, suggesting that this species has low rates of postmetamorphic growth. Herein, we study the development of the skeleton during larval development up to the end of metamorphosis, which is denoted by the complete lost of the tail in *P. platensis*. Our study revealed heterochronic differences in skeletal development compared with that of most anurans; these involve the complete differentiation of skull bones and the extensive ossification of the postcranial skeleton before completion of metamorphosis. The skull of metamorphosing *P. platensis* has an ossified sphenethmoid and a fully formed plectral apparatus, thus differing with regard to the pattern observed in most anurans in which both developmental events take place during the postmetamorphic life. Despite the fact that the iliosacral articulation and the urostyle are present at the end of metamorphosis as in most anurans, ossification/calcification of carpus, tarsus, and limb epiphyses during metamorphosis of *P. platensis* suggests that the postcranial skeleton lacks postmetamorphic growth. This study also includes a discussion of the pattern of development of the plectral apparatus, which allows us to propose a new hypothesis regarding pars externa plectri homology. J. Morphol. 270:205–220, 2009. © 2008 Wiley-Liss, Inc.

KEY WORDS: anura; *Pseudis*; osteology; heterochrony; development; tadpole; metamorphosis

The genus *Pseudis* comprises 11 species of aquatic frogs from the lowlands of Guianas, Northeastern Venezuela, Trinidad, Southern Brazil, Paraguay, Southeastern Peru, Eastern Bolivia, Northeastern Argentina, and Uruguay (Aguiar et al., 2007; Frost, 2007). The species of *Pseudis* possess elongated intercalary elements, belong to Hylidae, the most diverse group of neotropical treefrogs, and are included in the clade Dendropsophini (Faivovich et al., 2005). The intercalary elements are formed by hyaline cartilage, differing from most hylodine anurans (Manzano et al., 2007). *Pseudis* displays complete webbed feet with a distinct paddle-like morphology, a consequence of isochronic development of Toe IV with respect to other digits. This feature is convergent with pipoids (Goldberg and Fabrezi, 2008). Furthermore, *Pseudis* exhibits a pattern of fingers in which Finger II is opposable to other digits (Laurent, 1986).

Some species formerly recognized as subspecies of *Pseudis paradoxa* are unique in the size of the adult frog compared with the size of the tadpole (Emerson, 1988). Since *P. paradoxa* was described by Linnaeus (1758), many aspects of the large tadpole have been mentioned as being different from those of other anurans. Larval development of *Pseudis platensis*, which has a giant tadpole, seems to be related to a long developmental time and dissociated developmental events, in which a "delayed" metamorphosis is characterized by late offset of some developmental events that are completed in froglets that are nearly the same size as that of sexually mature adults (Fabrezi and Quinzio, in press). The larval development of *P. platensis* also suggests that some morphological changes linking the larval and adult body plans (e.g., skull configuration) could be affected by developmental perturbations of consequence during the postmetamorphic ontogeny of this taxon. Emerson (1988) suggested that *P. paradoxa* can metamorphose at a length very close to that seen in a sexually mature frog, although this does not necessarily imply the loss of posmetamorphic growth and a shift in the absolute time to sexual maturity. In *P. platensis*, size at metamorphosis approaches the mean length of adults, and data on skeletochronology revealed that adults have two or three lines of arrested

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growth, thereby suggesting this species is not long-lived (Fabrezi and Quinzio, in press). A giant tadpole, long larval development, large size at metamorphosis, and dissociated developmental events in the species of *Pseudis* are special features that offer the possibility to explore aspects about the morphological evolution of anuran metamorphosis.

Anuran skeletal morphology has received considerable attention and is one of the most important issues contributed to our understanding of anuran systematics and phylogeny. The anuran larval skeleton has been the focus of different studies that provided evidence to explain morphological variation (e.g., Maglia et al., 2001; Haas, 2003), describe metamorphic transformations (e.g., de Jongh, 1968; Wiens, 1989; Hall and Larsen, 1998), and interpret homology (e.g., Jarošová, 1974; Fabrezi and Alberch, 1996; Pugener and Maglia, 2007). Thus, available information on anuran skeletal morphology is sufficient to allow comparisons and detect developmental variation in taxa having unusual ontogenetic features, such as *P. platensis*.

Despite the many noticeable features of *P. platensis*, the skeletal ontogeny of this taxon has not been described; hence, we lack a context to seek an understanding of the significance of the huge tadpole, delayed metamorphosis, and dissociated developmental events and the bearing these phenomena might have on the configuration of the adult skeleton. The following account provides baseline descriptive data of larval development of the skeleton in *P. platensis*. We consider metamorphosis to be complete when the tail is completely lost (as do Nieuwkoop and Faber, 1956; Gosner, 1960; Duellman and Trueb, 1986; Elinson et al., 1999). Comparisons of the developmental sequences and metamorphic events of *P. platensis* with data recorded for other species allow detection of developmental perturbations in different skeletal traits and provide new interpretive insights into delayed metamorphosis of this species.

MATERIALS AND METHODS

We examined a sample of 70 specimens of *P. platensis* (Gallardo, 1961) collected from November to April 2004–2007 in ephemeral ponds along National Route 81 (23°10′–14′S, 63°21′–39′W) in San Martin Department, Salta (Argentina). Specimens were fixed in 10% formalin in the field, and were deposited in the Herpetological Collection of the Museo de Ciencias Naturales (MCN), Universidad Nacional de Salta (Argentina), with the following numbers: 964 (December 2004), 968 (December 2004), 972 (December 2004), 973 (December 2004), 988 (February 2005), 1012 (December 2005), 1015 (March 2004), 1038 (November 2005), 1055 (March 2005), 1060 (February 2005), 1110 (November 2006), 1114 (February 2005), 1130 (March 2004), 1137 (March 2004), 1138 (March 2006), 1139 (April 2005), 1142 (April 2005), 1143 (April 2005), 1171 (March 2006), 1176 (March 2005), 1181 (December 2004), 1182 (February 2005), 1183 (February 2005), 1196 (March 2007), and 1197 (April 2007).

We selected 70 specimens forming a larval series from hind limb bud stages up to complete absence of tail, and 15 adults. Larval development before metamorphosis was staged according to

the developmental table of Gosner (1960). The beginning of metamorphosis was staged from forelimb emergence and the end of metamorphosis with complete tail loss. Adults were selected for having secondary sexual characters and/or mature gonads (males with vocal sacs and females with oviductal oocytes).

The study of skeletal variation was conducted using cleared and double-stained specimens following the method of Wassersug (1976), in which cartilage is stained blue with Alcian Blue and bone is stained red with Alizarine Red S. Histological preparations of Digits IV (hand and foot) were obtained for analysis of intercalary element development in larval specimens at Gosner's Stages 38–41. Histological serial sections (6 μm thick) of paraffin-embedded digit tips were stained with Alcian blue-PAS-hematoxylin. Histological sections of digit tips of *P. platensis* were compared with those of *Scinax fuscovarius* (MCN 1191) and *Phyllomedusa sauvagii* (MCN 1159) at similar developmental stages.

Observations and illustrations were made with the use of Nikon SMZ 800 stereomicroscope with attached camera lucida and 8.1 megapixel digital camera, and Leica DM EP light microscope.

Terminology and criteria for identifying skeletal structures follow those of Wiens (1989), Trueb and Hanken (1992), Trueb (1994), Fabrezi and Alberch (1996), Haas (2003), and Pugener et al. (2007).

RESULTS
Development of the Cranium and Hyoid Apparatus

The larval cranial skeleton and hyobranchial apparatus of *P. platensis* have been described by Alcalde and Barg (2006) in a revision of morphological features of *Pseudis* spp. tadpoles. In our sample, the cranial skeleton varies among larval Stages 31–41 with respect to the differentiation of the lateral walls of the neurocranium, tectum parietal, planum internasalis, and taenia ethmoidalis which are fully formed at Stage 36 (Fig. 1A,B). The first ossifications to appear are the dermal parasphenoid and frontoparietals (Stage 37), and thereafter, the endochondral prootics and exoccipitals mineralize (Fig. 1C). The hyobranchial skeletal features described by Alcalde and Barg (2006) do not change after metamorphosis begins (Fig. 2A).

Before forelimb emergence (Stage 41) the neurocranium starts to display transformations which are evident in: 1) differentiation of cartilages of the olfactory capsules (superior prenasal cartilage, alary cartilage, oblique cartilage), paries nasi/crista subnasalis, and growth of the posterior nasal wall and lamina orbitonasalis, which separate the fenestrae nasolateralis and endonarina communis; and 2) erosion of the medial union of the partes corpores of suprarostral cartilage. Furthermore, the ossifications of prootic and exoccipital become confluent, forming an extensive ossification of the laterointernal wall of the otic capsule. Each premaxilla appears attached to the superior prenasal cartilage, and both appear overlapped the trabecular horn. The septomaxilla is also differentiated and contiguous to the alary cartilage. The posterior margin of the fenestra nasolateralis is covered by the nasal ossification. The
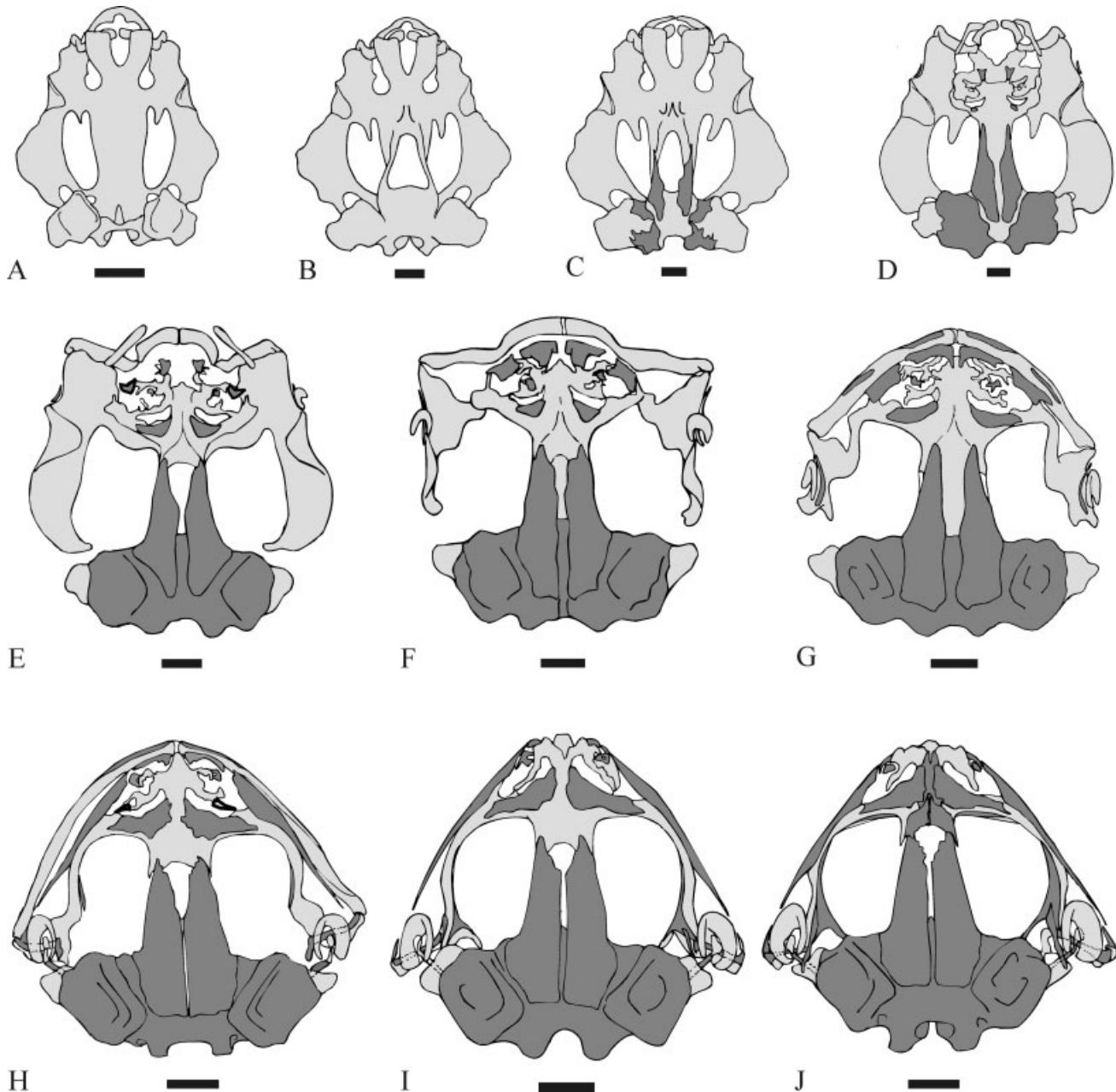
Fig. 1. Dorsal views of skull development during larval and metamorphic stages of *Pseudis platensis*. Dark gray areas indicate skull ossifications. **A**: Stage 32. Chondrocranium of an early tadpole. Only the floor of the braincase is cartilaginous (see description in Alcalde and Barg, 2006). **B**: Stage 36. The larval cartilaginous skull is completely formed. The braincase is closed by lateral cartilages, and dorsal (tectum nasi, tectum parietal) structures (see description in Alcalde and Barg, 2006). **C**: Stage 40. Dermal ossifications of parasphenoid (ventral) and frontoparietals, and the endochondral prootics and exoccipitals are developed. **D**: Differentiation of the dorsal cartilages of the olfactory region and dermal ossifications (premaxillae, maxillae, septomaxillae, squamosals) at the beginning of metamorphosis. **E**: Erosion of larval structures (suprarostral cartilage and trabecular horns). Differentiation of the tympanic annulus. **F**: Beginning of lower jaw elongation. Erosion of posterior processes of palatoquadrate. **G**: Fusion of the nasal cartilages (lamina orbitonasalis and posterior nasal wall) with the palatoquadrate. **H**: The pterygoideus processus and the quadrate reach the otic capsule. **I**: The adult suspensorius is formed. The plectral apparatus is complete. **J**: At the end of metamorphosis the skull is fully ossified, and could be considered an adult skull. No bone is reduced or absent in this species. Scale bar equals 2 mm.

ventral ramus of the squamosal appears as a thin ossified bar placed horizontally along the border of the palatoquadrate, immediately ahead of the base the processus muscularis (Fig. 3A).

At the time of the emergence of forelimbs, the erosion of the partes corpores of suprarostral cartilage progresses, the paranasal commissure extends between the oblique cartilage and the postnasal
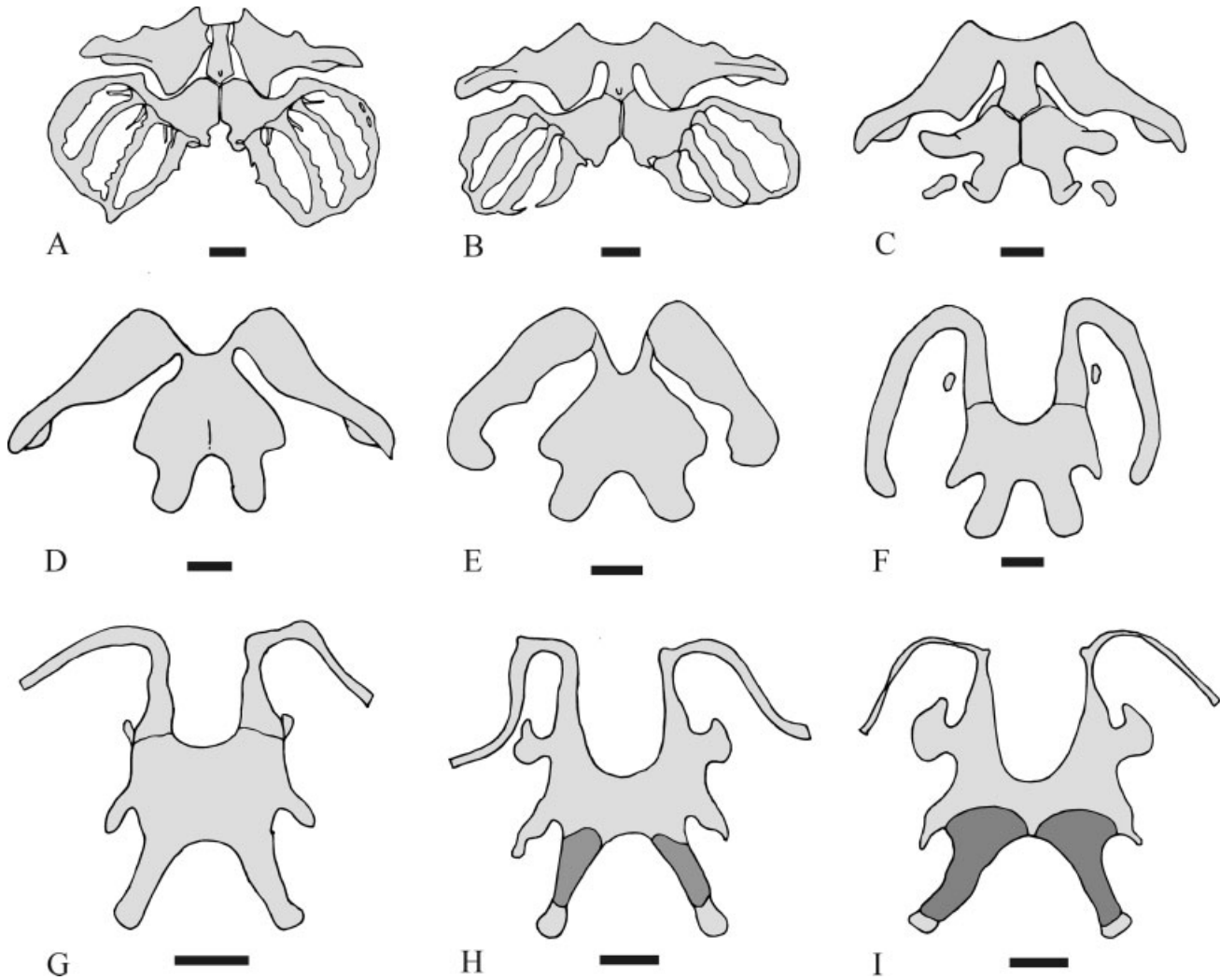
Fig. 2. Ventral views of hyobranchial apparatus transformations during larval and metamorphic stages of *Pseudis platensis*. Dark gray areas indicate ossifications. **A**: Hyobranchial skeleton during the larval period (Stages 30–41) (see description in Alcalde and Barg, 2006). **B**: Disto-proximal erosion of the cartilages of the branchial basket at the beginning of metamorphosis. **C–E**: Metamorphic transformations affect the shape of the ceratohyalia, hypobranchialia, copula, and pars reuniens. **F**, **G**: Appearance of *de novo* condensations that fuse with the ceratohyalia to form the anterolateral processes. **H**, **I**: Ossification of the posteromedial processes at the end of metamorphosis. Scale bar equals 2 mm.

wall and lamina orbitonasalis, closing the fenestra nasolateralis (Fig. 1D). In the hyobranchial apparatus (Fig. 2B), the spiculae and commissure terminalis between Ceratobranchialia IV and III start to be eroded. This erosion proceeds rapidly in the disto-proximal direction on each ceratobranchial.

Concomitant with the disappearance of keratinized jaw sheaths of the oral disc, the total degeneration of both partes corpores of suprarostral occurs while the partes alaris remain united medially by soft tissues with weak AB coloration (Fig. 1E). Simultaneously, the trabecular horns are reduced to their proximal vestiges (Fig. 1E). At this stage, the hyobranchial apparatus shows major transformations (Fig. 2C): each ceratohyal moves and the process anterior appears displaced

forward as a consequence of the enlargement of the impair copula and pars reuniens; each hypobranchial has two processes (lateral and posterior) resulting from reduction of Ceratobranchialia I and IV, respectively. There are vestiges of Ceratobranchial III without connections with the apparatus.

While the keratinous teeth are lost from the oral disc, the lower jaw increases in length and becomes more transversely oriented, and the larval upper jaw is conserved as free vestiges of the partes alaris of the suprarostral cartilage (Fig. 1F). The larval processes pseudopterygoideus, ascendens, and oticus of palatoquadrate disappear. With the loss of the latter two structures, the palatoquadrate loses its posterior attachment to the cranium. The attachment of the commissure quad-
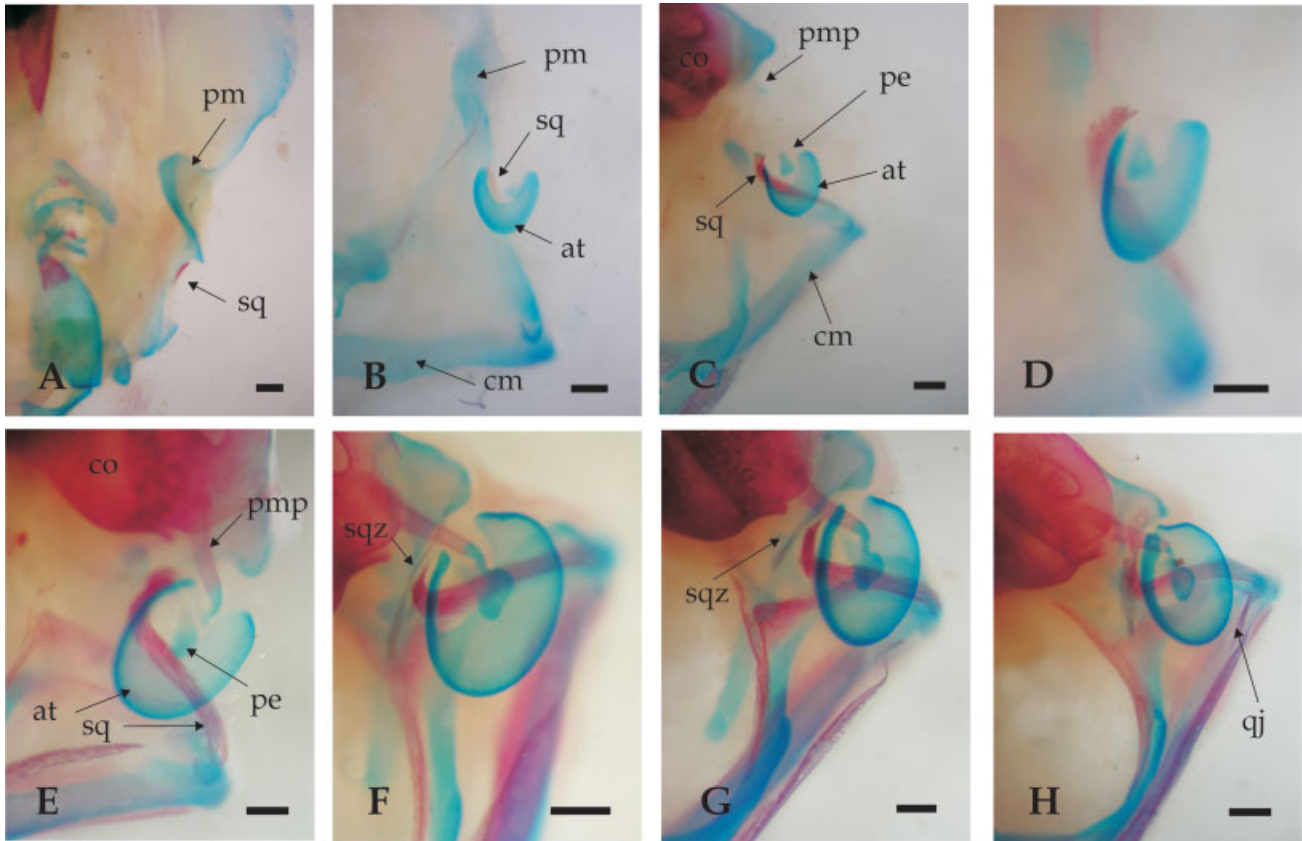
Fig. 3. Differentiation of the plectral apparatus in *Pseudis platensis* during its metamorphosis. **A**: Differentiation of the ventral ramus of squamosal immediately anterior to processus muscularis of palatoquadrate. **B**: The tympanic annulus arises as a semilunar cartilage lateral to the ventral ramus of the squamosal. **C**: The pars externa plectri appears embraced by the sickle-like tympanic annulus. The ventral ramus of the squamosal grows and turns 70°. The cartilaginous pars media plectri attaches to the fenestra ovalis and is oriented lateroexternally. **D**: The same of C, in detail. **E**: The pars media plectri is ossifying. **F**: The posterior attachment of the upper jaw and neurocranium is formed. The ventral ramus of the squamosal has moved backward. The pars media plectri is already ossified and reaches the pars externa, which is embraced by the incomplete ring of the tympanic annulus. The pars externa plectri overlaps the ventral ramus of the squamosal. **G**: The dorsal arm (zygomatic and otic rami) of the squamosal differentiates as a distinct ossification from the ventral ramus. **H**: The zygomatic and otic rami and the ventral ramus are fused, and the squamosal is formed. The plectral apparatus is fully formed. at, tympanic annulus; cm, Meckel's cartilage; co, otic capsule; pe, pars externa plectri; pm, processus muscularis of palatoquadrate; pmp, pars media plectri; qj, quadratojugal; sq, ventral ramus of the squamosal; sqz, zygomatic and otic rami of the squamosal. Scale bar equals 1 mm.

ratocranialis anterior to the floor of the braincase starts to erode. The nasal septum extends forward the anterior nasal wall to protrude as the median prenasal process (Fig. 1F). At this stage, the tympanic annulus differentiates as a semilunar cartilage lateral to the ventral ramus of the squamosal (Fig. 3B). In the hyobranchial apparatus (Fig. 2D), the anterior and posterior processes of ceratohyal are less evident, and the ceratohyal tapers gradually, although the condylus articularis is still present. The hyoglossal sinus is deeper, and there is a thin cartilaginous connection between the ceratohyal and the incipient hyoid plate (formed by the fused hypobranchialia that have incorporated the copula and pars reuniens).

During the next step of development, the infrarostral cartilage and Meckel's cartilages are fused and enlarged, and the jaw suspension is displaced laterally (Fig. 1G). There are no vestiges of the suprarostral cartilage and trabecular horns. The commisure quadratocranialis anterior becomes slender, and turns below the end of the planum triangulare, which bears the well differentiated anterior and posterior maxillary processes. The commisure quadratocranialis fuses to the planum triangulare. The palatoquadrate undergoes extensive erosion at the level of the processus muscularis. The pars facialis of the maxilla is well defined. The alary process and the pars dentalis of the premaxilla are distinct. The ventral ramus of the squamosal lengthens and retains its horizontal position. The pars externa plectri lies within the sickle-shaped tympanic annulus. Changes in the hyobranchial apparatus occur in the ceratohyal, which is uniform in width and bears vestiges of the condylus articularis recognizable in its

rounded distal tip (Fig. 2E). There is a suture between the ceratohyal and the hyoid plate.

Subsequent development involves elongation of the lower jaw and posterior displacement of its articulation, simultaneously with elongation of the pterygoideus processus formed by the cartilaginous material of the larval palatoquadrate (Fig. 1H). The ventral ramus of the squamosal begins to turn downward (Fig. 3C,D). The dermal ossifications of the vomer and pterygoid are differentiated. The pterygoid, present as the anterior ramus, is attached to the posterior end of the pterygoideus processus. In the lower jaw, the dentary and angulosplenial are developed. The cartilaginous pars media plectri emerges from the fenestra ovalis and orients lateroexternally (Fig. 3C). In the hyoid apparatus (Fig. 2F) the ceratohyal becomes slender, longer, curved, and limits the deep hyoglossal sinus. The precursor of the anterolateral process of the hyoid is differentiated as a *de novo* cartilage that appears contiguous and lateral to each ceratohyal, near the suture between the ceratohyal-hyoid plate. The posterolateral process is present in the place where Ceratobranchial I was connected to the hypobranchial and the posteromedial process is differentiated in the Ceratobranchial IV position.

When the vent tube is almost absent and the tail starts to reduce, the skull displays the posterior attachment between the pterygoideus processus and the neurocranium (Fig. 1H). The ventral ramus of the squamosal has moved 70° (Fig. 3E). The pars media and pars interna plectri are already ossified and reach the pars externa plectri which is embraced by the incomplete ring of the tympanic annulus (Fig. 3E). The pars externa plectri overlaps the ventral ramus of the squamosal (Fig. 3E). The cartilaginous nasal capsules occupy the most anterior region of the skull and the median prenasal process separates the alary processes of premaxillae. Teeth are visible in the premaxillae, maxillae, and vomers. Each vomer has the pre- and postchoanalis processes differentiated. The pars facialis of the maxilla grows posteriorly. The pterygoid only exhibits its anterior ramus. In the hyoid apparatus (Fig. 2G), the cartilage of the anterolateral process is attached to the base of the ceratohyal.

At the stage when metamorphosing specimens have reduced tails, skull ossification is advanced (Fig. 1I). The three rami of the pterygoid are differentiated. The palatine and the quadratojugal arise. The quadratojugal is a small ossification in front of the ventral ramus of the squamosal and does not contact the maxilla. The dorsal arm (zygomatic-otic rami) of the squamosal differentiates as a distinct ossification from the ventral ramus (Fig. 3G). The pars dentigerous and pars anterior of the vomer are well differentiated. The bifid cultriform process of the parasphenoid reaches the solum nasi. The angulosplenial ventrally invests the lower jaw cartilage and the dentary covers its labial face. The hyoid apparatus is almost fully formed (Fig. 2H). The anterolateral process is incorporated into the ceratohyal and expands. The posterolateral process is pointed. Ossification in the posteromedial processes is advanced and reaches the limits of the hyoid plate.

During completion of metamorphosis, the tail is reduced to a stub and the complete set of skull ossifications is present (Fig. 1J). The septum nasi is mineralized. The sphenethmoid is also completely ossified. The quadratojugal contacts the maxilla. The zygomatic-otic rami of the squamosal and the ventral ramus are fused (Fig. 3H). The hyoid is similar to that of the adult (Fig. 2I). The posteromedial process is short and robust and its ossification is extended into the hyoid plate. The ceratohyal bears a tiny anterior process.

At the end of metamorphosis and in adults, skull ossification is extensive and cartilaginous areas remain in the nasal cartilages and the tympanic annulus, which is a complete ring. In ventral view the nasal floor is fully mineralized.

Development of Axial Skeleton

Nine vertebrae develop caudally during early of development (Stage 30). The cartilaginous paired neural arches close gradually (Stages 31–35). Vertebrae grow and ossification centers start to differentiate rostro-caudally and dorso-ventrally from the neural arches (Stages 36–41). Only Vertebrae III and IV exhibit well defined and short transverse processes. The cartilaginous coccyx (postsacral element) arises as paired element with two pairs of foramina that suggest it is formed by two postsacral vertebrae (from Stage 36). Simultaneously, the cartilaginous impair hypochord appears as a slender bar in the aponeurosis where the hypaxial musculatures meet. Before forelimb emergence (Stage 41) the elements of the vertebral column, including the hypochord, are well ossified. When metamorphosis begins, a progressive differentiation of transverse processes is evident, especially the processes of the sacral vertebra. At later stages of metamorphosis, the iliosacral articulation is formed and the hypochord ascends to fit into the ventral cavity of the coccyx. The fusion of the coccyx with the hypochord to form the urostyle occurs at the end of metamorphosis, simultaneously with complete resorption of the tail.

Development of the Appendicular Skeleton

Pectoral girdle and sternum. Each half of the pectoral girdle is composed of three free cartilaginous primordia that were differentiated in the sequence: scapula, coracoid, and procoracoid (Stages 31–34). They fuse to delimit the glenoid fossa (Stage 35). The suprascapular cartilage is

first visible as a distal process of the scapula (Stage 37). During Stages 38–41 the epicoracoid appears and forms a cartilaginous bridge between the coracoid and the procoracoid. Endochondral ossification of the coracoid and scapula occurs simultaneously with differentiation of the ossification of the clavicle and cleithrum. Soon after forelimb emergence, two divergent cartilaginous condensations become visible posterior to the epicoracoid cartilages. These will form the sternum. The cartilaginous omosternum develops between the anterior ends of the clavicles. Sternum and omosternum seem to be attached by ligaments to the elements of the girdle (procoracoids and epicoracoids). The adult pectoral girdle and the sternum are already formed at the early stages of the metamorphosis. The osseous elements are joined by synostosis. The sternum is a distally bifurcated cartilaginous plate and the omosternum is a small, distally expanded cartilage.

Forelimb. Forelimb morphology in the adult of *Pseudis platensis* is characterized by the presence of the humerus, radio-ulna, radiale, ulnare, Element Y, Distal Carpale 5-4-3 articulating with Metacarpalia V–III, Distal Carpale 2 articulating with Metacarpalia III and II, phalanges arranged in the typical formula 2-2-3-3, the intercalary elements, and the prepollex with two elements (Fig. 4A). Adult carpal morphology and its development in *P. platensis* have been already described by Fabrezi (1992, 2001) and Fabrezi and Barg (2001).

The sequence of limb development is depicted in Figure 4B–Q. The embryonic ulnare displays a preaxial process that was interpreted as the intermedium that never segments (Fabrezi and Alberch, 1996; Fabrezi and Barg, 2001) (Fig. 4B–D), the adult radiale is formed by the secondary fusion of two condensations (Stages 38 and 39) (Fig. 4B–J), and the Element Y results from the fusion of three single cartilages in Stage 37 (Fig. 4F,G). Concomitantly, the crista ventralis starts to differentiate on the humerus. At Stage 39, the last fusion of carpal elements (Distal Carpale 5-4 plus Distal Carpale 3) occurs (Fig. 4L). The ossification begins in Stage 38 and takes place proximo-distally and preaxially (Fig. 4J–Q). By the beginning of metamorphosis all diaphyses are ossified (humerus, radio-ulna, metacarpalia, and phalanges), and carpal elements initiate their mineralization together with the long bone epiphyses (Fig. 4O,P). The forelimb is completely ossified before the end of metamorphosis (Fig. 4Q).

Pelvic girdle. The ilium and pubis are visible as two free cartilaginous condensations at Stage 31. Soon after, they fuse, leaving an incomplete concave surface that will form the acetabulum where the femur fixes. During Stages 35–37 the ilium gets longer. As development progresses, the ilial shaft begins to ossify, simultaneously with the diaphysis of the femur. Each pubis attaches medially and the ischium appears. Before the beginning of metamorphosis, the cartilaginous ischium-pubis completes the acetabulum. Ossification in the ischium-pubis starts during early metamorphosis. Immediately, the three components of the pelvic girdle form a single ossified structure. Nevertheless, a suture between the ilium and the ischium is still present at the end of metamorphosis. The ilial shaft lacks dorsal ridge.

Hind limb. The *Pseudis platensis* hind limb is composed of the femur (without crest), tibio-fibula, fused tibiale and fibulare, Distal Tarsale 3-2, Distal Tarsale 1, Element Y, Metatarsalia V–I, phalanges arranged in the typical formula 2-2-3-4-3, intercalary elements, and the prehallux formed by two elements (Fabrezi, 1993, 2001) (Fig. 5A).

The sequence of limb development is depicted in Figure 5. By Stage 35, in the tarsus there are four distinct condensations distal to the enlarged proximal tarsalia (fibulare-tibiale): The two condensations in the postaxial position are transient and become incorporated into the end of the fibulare (Fig. 5E–G). These condensations could be interpreted as the Distal Tarsalia 5 and 4 that do not segment (see the "Discussion" section). The anterior condensations represent the Distal Tarsale 3 that will give rise in continuity the Distal Tarsale 2 (Fig. 5E), and the first condensation of the Element Y, which will be formed by two primary condensations (Fig. 5E–M). The Distal Tarsale 1 and the prehallical cartilages arise as single condensations, before digits are fully formed (Fig. 5I,J).

Ossification of diaphyses begins in the femur at Stage 37 slightly before ossification in the forelimb, and it occurs in a proximo-distal and postaxial-preaxial direction (Fig. 5J–Q). Before forelimb emergence, all diaphyses of metatarsalia and phalanges are ossified (Fig. 5O). In the epiphyses, mineralization begins during metamorphic periods (Fig. 5P). Tarsal bones are the last ones to initiate mineralization. By the end of metamorphosis the whole limb is ossified (Fig. 5Q), with the exception of the tip of the distal prehallical element that remains cartilaginous.

Intercalary elements. In both limbs, condensations of terminal phalanges appear by Stage 38, leaving a gap between them and the penultimate phalanges where the intercalary elements will differentiate (Fig. 6A–C). Different from the phalanges, which are already composed of hyaline cartilage, the intercalary elements develop from embryonic connective cells that begin to aggregate in the gap (Fig. 6D,E). Immediately, this embryonic connective tissue becomes hyaline cartilage, which is characterized by a basophilic matrix and numerous chondrocytes, in mitosis, situated in lacunae (Fig. 6E). This aggregate acquires the typical shape of the intercalary of *P. platensis* (cylindrical) (Fig. 6B,C). Later, when terminal phalanges are almost ossified, the intercalary elements exhibit
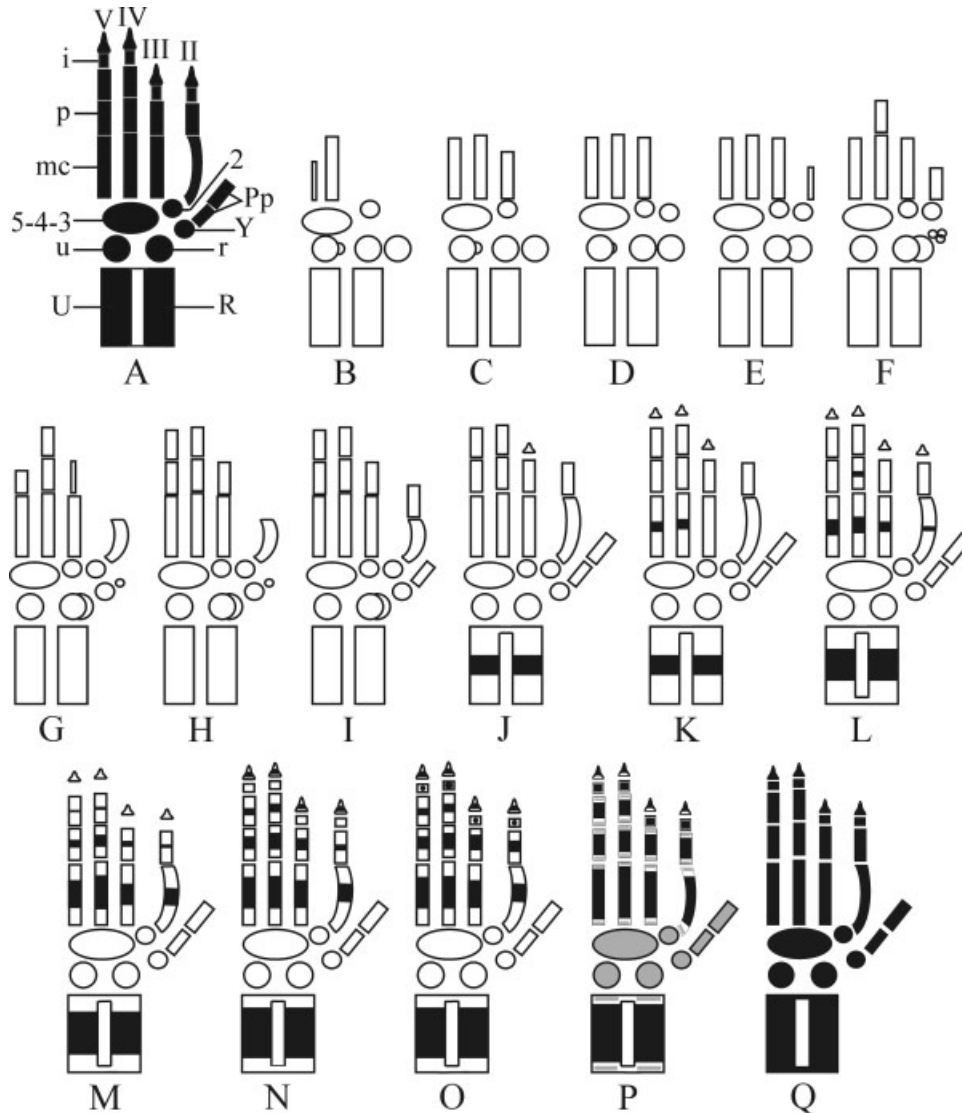
Fig. 4. Schematic sequence of chondrification and ossification of forelimb elements in *Pseudis platensis*. White color indicates cartilaginous elements; black color denotes ossified elements. Gray color represents ossification/calcification of epiphyses, carpus, and tarsus. **A**: Adult specimen. **B**: Stage 34. Cartilaginous condensations of the primary axis (Distal Carpalia 5-4 and Metacarpale IV) are well defined, as well as other carpal elements (ulnare, radialia, Distal Carpale 3) and Metacarpale V. The ulnare has a preaxial process, the intermedium. **C**: Stage 34, The Metacarpale III appears. **D**: End of Stage 34. Distal Carpale 2 becomes visible. **E**: Stage 35. The Metacarpale II arises. **F, G**: Stages 36 and 37. Sequence of differentiation of phalanges of Fingers V, IV and III, cartilages Y^1, Y^{11}, Y^{111} (which then fuse in a single Element Y), and the proximal element of the prepollex. Metacarpalia V, IV, and III are parallel, and articulate ventro-distally with Distal Carpals 5-4 and Distal Carpale 3, respectively. Metacarpale II curves and articulates by the preaxial side with Distal Carpale 2. **I–L**: Stages 38 and 39. Distal Carpale 3 fuses with Distal Carpal 5-4, and all remaining phalanges and the distal prepollical element appear. Ossification of diaphyses progresses in a proximo-distal and postaxial-preaxial direction. **M, N**: Stages 40 and 41. Terminal phalanges acquire their final straight and pointed shape and the intercalary elements arise. **O, P**: Subsequent events during the metamorphosis involve the ossification/mineralization of intercalary elements, epiphyses and carpal bones. **Q**: Full ossification of all elements precedes the complete loss of the tail. 2, Distal Carpale 2; 5-4-3, Distal Carpale 5-4-3; II–V, Fingers II, III, IV, and V; i, intercalary elements; mc, Metacarpalia; p, phalanges; Pp, prepollex; R, radius; r, radiale; U, ulna; u, ulnare; Y, Element Y.

fewer (although numerous) but larger chondrocytes, which continue dividing, in a more abundant matrix (Fig. 6F). The hyaline cartilage of the intercalary elements mineralizes simultaneously in both limbs. Mineralization starts at the beginning of metamorphosis.

DISCUSSION
Development of the Cranium and Hyoid Apparatus

Skull metamorphosis in anurans involves major changes in the ethmoidal region and jaws of the larval cranium. The earlier events of chondrocranial
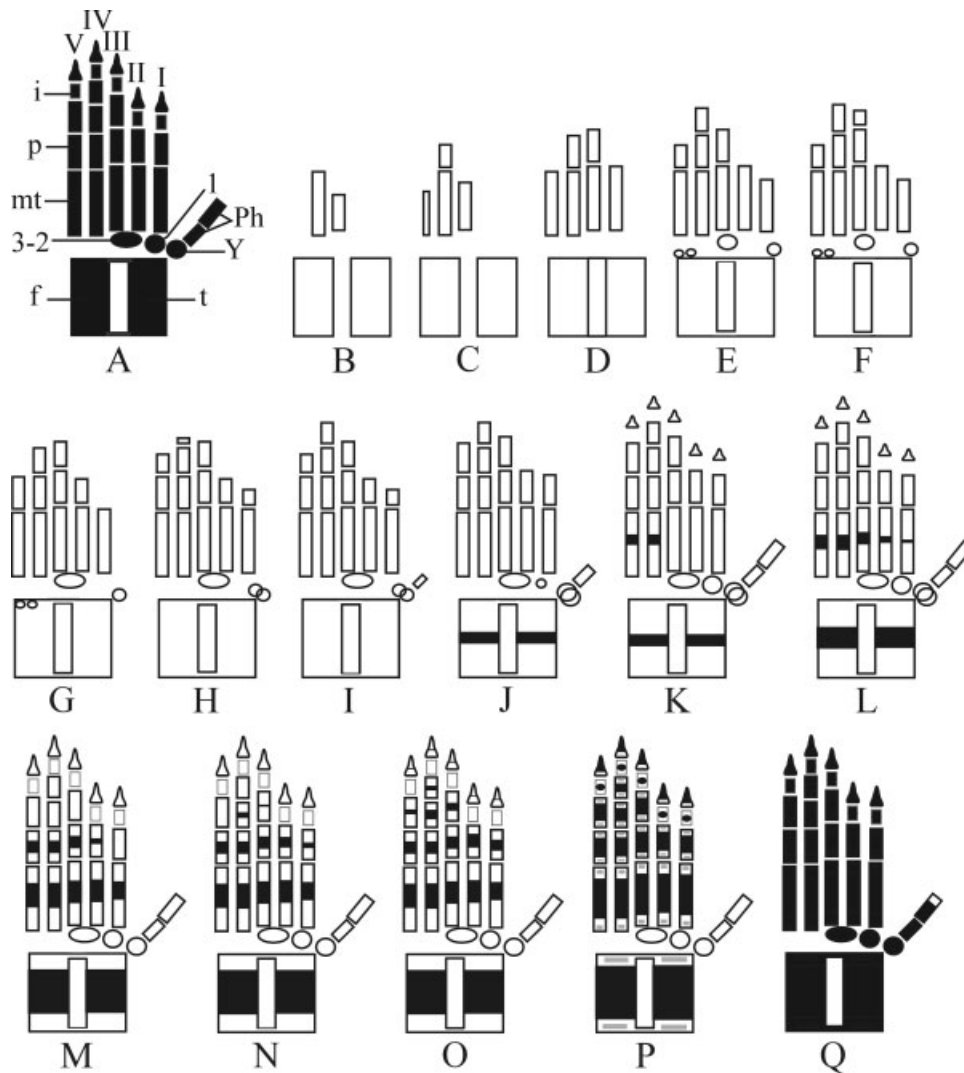
Fig. 5. Schematic sequence of chondrification and ossification of hind limb elements in *Pseudis platensis*. White color indicates cartilaginous elements; black color denotes ossified elements. Gray color represents distinctive ossification/calcification of epiphyses, carpus, and tarsus. **A**: Adult specimen. **B**: Stage 31. Condensations of primary axis (femur, fibula, fibulare, Metatarsale IV) are well developed, as well as the tibia, tibiale, and the primordium of Metatarsale III. **C**: Stage 33. The condensation of Metatarsale V appears together with the condensation of the proximal phalanx of Toe IV. **D**: Stage 34. Condensations of Metatarsale II and proximal phalanx of Toe III are visible. By the end of this stage the fusion of the distal ends of the tibiale and the fibulare begins. **E–G**: Stage 35. The tibiale and fibulare are completely fused at both ends. Metatarsale I appears together with the proximal phalanx of Toe V and the second phalanx of Toes IV and III. Tarsal condensations (Distal Tarsale 3 and Element Y) are visible. **H**: Stages 35 and 36. A second condensation of the adult Element Y (Y') differentiates. **I**: Stage 36. The proximal element of the prehallux and proximal phalanges are formed. **J**: Stage 37. Distal Tarsale 1 originates as a single condensation between the proximal ends of Metatarsalia I and II. **K**: Stage 38. Subsequent development involves the differentiation of the distal element of the prehallux and distal phalanges. **L**: Stage 39. Ossification of diaphyses progresses in postaxial-preaxial direction. **M**: Stage 40. The fusion of condensations of Element Y, and the appearance of intercalary elements occur. **N, O**: Stage 41. Ossification in diaphyses progresses in proximo-distal and postaxial-preaxial direction. **P**: Metamorphic events involve the ossification/mineralization of intercalary elements and epiphyses followed by the tarsal bones. **Q**: At the end of metamorphosis all elements are fully ossified. 1, Distal Tarsale 1; 3-2, Distal Tarsale 3-2; I–V, Toes I–V, f, fibulare; i, intercalary element; mt, metatarsalia; p, phalanges; Ph, prehallux; t, tibiale; Y, Element Y.

metamorphosis start with the erosion of suprarostral cartilage and trabecular horns, followed with the resorption of the posterior attachments of the palatoquadrate to the neurocranium (larval process oticus and process ascendens). Simultaneously, the complete differentiation of dorsal cartilages of the olfactory region—that form as *de novo* condensations (Pugener and Maglia, 2007)—, elongation of the lower jaw, and the posterior displacement of the pars articularis of palatoquadrate occur. In *P. platensis* these changes display the same patterns observed in
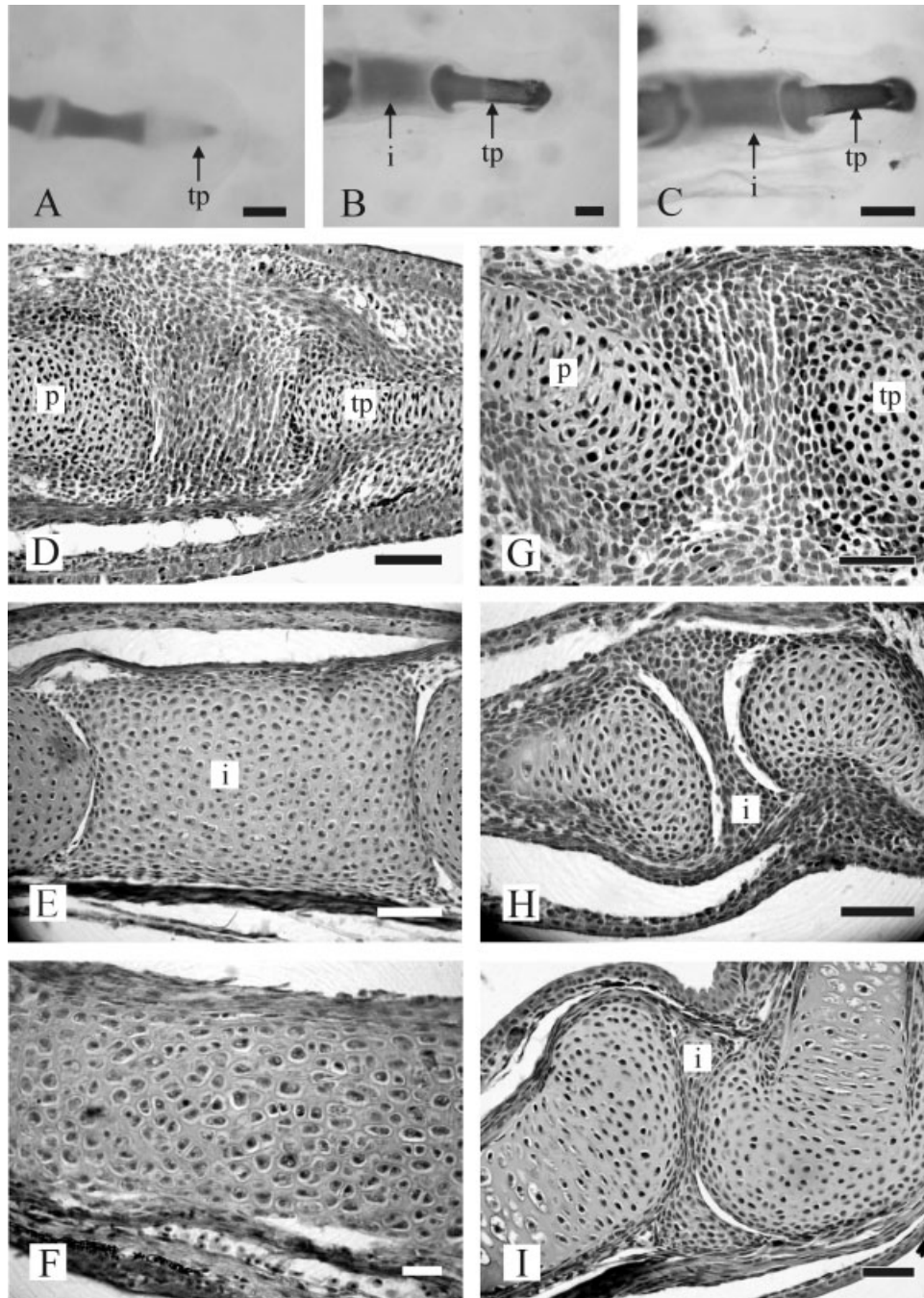
Fig. 6.   Intercalary element development in *Pseudis platensis* and other hylids. **A–F**: *Pseudis platensis*, Stages 38–41. A–C: Intercalary elements in whole-mount specimens are first visible when the terminal phalanges have begun their ossification. D–F: Longitudinal sections of Finger IV tip showing the embryonic cartilage (in D) and the hyaline cartilage (E, F) that will form the intercalary element. **G–I**: Development of intercalary element in *Scinax fuscovarius* and *Phyllomedusa sauvagii*. Intercalary elements also appear after terminal phalanges have differentiated (Stages 40 and 41) but remain as a condensation of embryonic cartilage that delimits a short biconcave disc. G: *Scinax fuscovarius*, Stage 38. H: *Scinax fuscovarius*, Stage 41. I: *Phyllomedusa sauvagii*, Stage 41. i, intercalary element; tp, terminal phalanx. Scale bar equals to 0.1 mm in A, B and F, 0.5 mm in C, and 0.05 mm in D, E, G–I.

other anurans (de Jongh, 1968; de Sá, 1988; Haas, 1999; Sheil, 1999; Perotti, 2001; Sheil and Alamillo, 2005; Pugener and Maglia, 2007). The sequence of ossification also exhibits similar features to those observed in most anurans, e.g., ossification of the frontoparietals, parasphenoid, exoccipitals, and prootics occurs before the beginning of metamorphosis, and the pterygoids, quadratojugals, palatines, and sphenethmoid are the last to differentiate.

There are two noteworthy and unique features of skull development in *P. platensis* relative to other anurans: the complete set of skull bones is well differentiated before the end of metamorphosis; and the development of the plectral apparatus and its early differentiation, already complete at the mid-metamorphosis.

The adult skull and hyoid apparatus of *P. platensis* are well ossified and present the complete set of dermal and endochondral bones typical of the neobatrachians (Trueb, 1994). No bone is lost or reduced in this species (e.g., vomer and squamosal are triradiate, the sound-conducting apparatus is complete, the palatine is present), and they are fully differentiated before the end of metamorphosis. Furthermore, teeth are also present in dentigerous bones (premaxillae, maxillae and vomers). In hylids, the sequence of ossification during metamorphosis was described for *Hypsiboas lanciformis* (de Sá, 1988) and *Phyllomedusa vaillantii* (Sheil and Alamillo, 2005), and postmetamorphic ossification was studied in *Acris crepitans* (Maglia et al., 2007). Quadratojugals, vomers, palatines, pterygoids, and the plectral apparatus appear after metamorphosis in *H. lanciformis*, and the palatines, quadratojugals, sphenethmoid, and plectral apparatus differentiate postmetamorphically in *P. vaillantii*. In *Acris crepitans*, a short postmetamorphic life may result in a small adult with reduced skull ossification. In other anurans in which skulls are well ossified and loss of bones does not occur, e.g., *Pyxicephalus adspersus* (Haas, 1999; Sheil, 1999), *Ceratophrys cornuta* (Wild, 1997), *Chacophrys pierottii* (Wild, 1999), *Leptodactylus chaquensis* (Perotti, 2001), and *Rana temporaria* (de Jongh, 1968), the palatines and sphenethmoid develop after metamorphosis, and complete differentiation and ossification of the components of the sound-conducting apparatus also occur after metamorphosis. These data suggest that the adult skull configuration and ossification in *P. platensis* is completed during metamorphosis, earlier than in most anurans.

The second special feature of skull development of *P. platensis* is the developmental pathway of the sound-conducting apparatus, which is already fully formed during metamorphosis. In anurans, the sound conducting apparatus is formed by two systems: 1) the tympanic system formed by the middle ear cavity, the tympanic membrane that is stretched over the tympanic annulus, and the stapes, that consists of a proximal footplate located anterior to the operculum in the fenestra ovalis, and a distal bony stylus (pars media plectri) that bears a cartilaginous connection (pars externa plectri) to the tympanic annulus; and 2) the opercular system, including the cartilaginous operculum and the opercularis muscle (Hetherington, 1988; Smirnov and Vorobyeva, 1988; Trueb, 1994). Reduction of the auditory system seems to follow an obligate sequence in which the tympanum and the tympanic annulus are the structures usually absent (Smirnov and Vorobyeba, 1988). The tympanum, the tympanic annulus, and the stapes are absent in some *Microhyla* spp., *Bombina*, *Ascaphus*, *Pelobates*, and *Rhinophrynus* (Smirnov and Vorobyeva, 1988; Trueb, 1994).

Table 1 summarizes the sequences of differentiation of the sound-conducting apparatus in anurans. Hetherington (1988) described the metamorphic changes in the middle ear of amphibians and stated that the general pattern of formation is consistent among anurans. The opercularis system shows little variation in timing of development, and it was always completed during metamorphosis and before the initiation of terrestrial activity (Hetherington, 1988). The tympanic system, however, displayed marked differences in timing of formation, and it may be quite underdeveloped by the end of metamorphosis (Table 1; Hetherington, 1988). In contrast, *P. platensis* and *Xenopus laevis* (Trueb and Hanken, 1992) share an inverse sequence in which the operculum is the last element to be differentiated and the only one that appears after metamorphosis. Among other functions, the opercular system has been associated with reception of substrate vibration and low-frequency sound, whereas the tympanic system acts in underwater hearing, sound localization, protection from loud sounds, and amplification (Jaslow et al., 1988). Hetherington (1988) found correspondence between the degree of development of the tympanic system and body size at metamorphosis: in small species, the tympanic system is very incomplete, and in species metamorphosing at slightly larger body sizes, development of the tympanic ear is farther along. This correspondence seems to explain the premature development of the tympanic system in *Pseudis* and *Xenopus* (because they include medium to large species), although it is absent in other large metamorphosing species that have an incomplete tympanic system at metamorphosis [e.g., *Ceratophrys* (Wild, 1997), *Pyxicephalus* (Haas, 1999; Sheil, 1999), and *Lepidobatrachus* (Fabrezi, unpublished data)]. Smirnov and Vorobyeva (1988) suggested that the opercular system appears first and the tympanic system usually progresses through postmetamorphic development, whereas its morphological differentiation may be delayed until sexual maturity. Thus, the functions of monitoring of environmental sounds (opercular system) and perception of specific reproductive information (tympanic system) may be combined in the tympanic system with its premature formation in some species. This interpretation implies the existence of two systems capable of combining both presumed functions, providing grounds for rather ready reduction or loss of one of these systems (Smirnov and Vorobyeva, 1988). Nevertheless, even when the tympanic system shows reduction among anurans, the acceler-

TABLE 1. *Comparisons of the sequence of the differentiation of the sound conducting apparatus among anurans*

Pseudis platensis	Amyetophrynus regularis	Pyxicephalus adspersus, Ceratophrys cornuta, Leptodactylus chaquensis, Hypsiboas lanciformis	Xenopus laevis	Pipa pipa	Spea bombifrons
Tympanic annulus	Operculum	Operculum	Tympanic annulus, pars externa, and pars media plectri	Pars media plectri	Operculum
Pars externa plectri	Cartilaginous pars interna plectri	Ossification of pars media plectri	Tympanic annulus forming an incomplete ring. Ossification of pars media plectri. Cartilaginous pars interna plectri	Tympanic annulus and pars externa plectri	Pars media plectri
Pars media plectri	Pars media and pars externa plectri. Tympanic annulus and tympanic membrane	Tympanic annulus	Operculum		
Ossification of pars media plectri. Association between the distal tip of pars media plectri and pars externa plectri	Ossification of pars media plectri			Ossification of pars media plectri	Ossification of pars media plectri, and operculum. Tympanic annulus
Footplate (pars interna plectri). Tympanic membrane	Annulus tympanicus forming a complete ring			Ossification of annulus tympanicus	Footplate (pars interna plectri)
Tympanic annulus forming a complete ring. Fusion of pars media plectri and pars externa				Operculum	Pars externa plectri
Operculum				Ossified tympanosquamosal	Tympanic annulus is an incomplete ring

Shaded cells correspond to those events that take place before the end of metamorphosis. Light gray areas indicate events that occur before the beginning of metamorphosis. Literature data: *Amyetophrynus regularis* (Sedra and Michael, 1959), *Hypsiboas lanciformis* (de Sá, 1988), *Spea bombifrons* (Wiens, 1989), *Xenopus laevis* (Trueb and Hanken, 1992), *Ceratophrys cornuta* (Wild, 1997), *Pyxicephalus adspersus* (Sheil, 1999; Haas, 1999), *Pipa pipa* (Trueb et al., 2000), and *Leptodactylus chaquensis* (Perotti, 2001).

ated pattern of development that produces completely formed tympanic systems during metamorphosis in *Pseudis* and *Xenopus* (Trueb and Hanken, 1992) does not involve loss or reduction in the opercular system. A functional explanation for this accelerated pattern could be associated with a specialization of the tympanic system to hear underwater.

The sequence and accelerated pattern of development of the tympanic system observed in *Pseudis* and *Xenopus* is not identical (Table 1). In *Xenopus laevis* external and middle ear elements appear abruptly during metamorphosis (Stage 62 of Nieuwkoop and Faber, 1956), when the anterior part of the palatoquadrate has moved backward and associates with the anterolateral corner of the otic capsule (Trueb and Hanken, 1992). This fact suggests the development of the sound conducting apparatus is temporally and spatially integrated after the massive changes in the suspensorium. In *Pseudis platensis*, temporal and spatial dissociation in the appearance of the tympanic annulus-pars externa plectri (related to the ventral ramus of squamosal primordium and the processus muscularis of palatoquadrate), and the pars media-interna plectri (associated to the fenestra ovalis) is in accordance with the proposed origin of the tympanic annulus from the quadrate, representing the dorsal quadrate process of dissorophids (Bolt and Lombard, 1985). de Beer (1937) noted that the operculum arises quite independently of the wall of the auditory capsule and for a short time acquires a cartilaginous continuity with the edge of the fenestra ovalis, whereas the origin of the stapes is homologized with the hyomandibular (de Beer, 1937; Lombard and Bolt, 1988). Descriptions of the stapes in anurans pointed out that the three parts of it develop in continuity (de Beer, 1937; Sedra and Michael, 1959; Hetherington, 1988). However, the development of the stapes observed in *P. platensis*, in which the pars extrena plectri differentiates closely to the tympanic annulus earlier and is separated from the pars media plectri suggests two hypotheses: 1) the pars externa plectri is formed by a *de novo* condensation and represents a novel anuran morphological feature; or 2) the embryological origin of the pars extrena plectri could be related to the tympanic annulus and the palatoquadrate rather than to the hyoid arch cartilages. In any case, these proposals emerge from the dissociated sequence that results from heterochrony in the formation of the tympanic system of *P. platensis*.

Development of the Axial and Appendicular Skeleton

In *P. platensis*, the events of development of the vertebral column follow the same overall pattern described in the literature. However, Ročková and Roček (2005) in a study of development in some taxa (*Discoglossus*, *Bombina*, *Bufo bufo*, *Rana dalmatina*, *Xenopus laevis*) mentioned that only in Pipidae (and Paleobatrachidae) the hypochord may even ossify separately from other components of the urostyle. This is similar to the situation observed in *P. platensis* and also in other taxa and apparently is a frequent condition among anurans (de Sá, 1988; Wiens, 1989; Pugener and Maglia, 1997; Wild, 1997, 1999; Hall and Larsen, 1998; Maglia and Pugener, 1998; Haas, 1999; Sheil, 1999; Perotti, 2001; Sheil and Alamillo, 2005). Similar to the case in most anurans, the fusion of the hypochord and the coccyx to form the urostyle is concomitant with the complete reduction of the tail.

The cartilages of the girdles start to differentiate almost simultaneously with the proximal elements of the limbs (humerus/femur) and the ossification occurs in old tadpoles before metamorphosis. In arciferal taxa, the epicoracoids overlap after the emergence of forelimbs, and soon after, the cartilaginous and paired condensations of the sternum become differentiated [except in *Spea bombifrons* in which the sternum develops from a single condensation (Wiens, 1989)]. In the pelvis, the iliosacral articulation is well defined at larval Gosner's Stages 43–45, when tail resorption is advanced. In *P. platensis*, the formation of the iliosacral articulation coincides with the complete loss of the vent tube, before the beginning of the events of tail resorption.

The morphogenesis of primary cartilages in limb development of *P. platensis* is similar to that described following the interpretations proposed by Shubin and Alberch (1986) in different anuran taxa (Schmalhausen, 1907; Fabrezi, 1992, 1993, 2001; Fabrezi and Alberch, 1996; Pugener and Maglia, 1997; Haas, 1999; Fabrezi and Barg, 2001; Perotti, 2001; Sheil and Alamillo, 2005; among others). Despite abundant information on early limb development in anurans, data on sequence of digit development and ossification patterns are limited (Wiens, 1989; Perotti, 2001). From our results, some aspects observed in *P. platensis* are worthy of discussion.

1. Hind limb chondrification begins earlier and progresses faster than forelimb chondrification despite the fact that hind and forelimb buds appear simultaneously in *P. platensis*. However, the ossification begins almost simultaneously in both limbs, when terminal phalanges have appeared (Gosner Stages 37 and 38), but finishes later in hind limbs.
2. The sequence of appearance of metacarpalia/metatarsalia and phalanges differs between fore and hind limbs. In forelimbs, metacarpalia develop in the sequence IV-V-III-II while meta-

tarsalia develop in the sequence IV-III-V-II-I. Different from most anurans, in *P. platensis* and *Xenopus laevis* the Metatarsale III appears almost synchronically with Metatarsale IV and before Metatarsale V, which is related to the development and conservation of a paddle-like foot in the adult (Goldberg and Fabrezi, 2008).

3. Differences between manus and pes are given by the sequence of phalanges differentiation. In the hand, phalanges appear after the four metacarpalia are differentiated and follow the sequence of metacarpalia development. In the foot, first phalanges differentiate before appearance of Metatarsalia II and I, and follow the sequence of metatarsalia development.

4. According to Shubin and Alberch (1986), in anurans Distal Tarsale 4 retains its connection with the fibulare, while Digit V could arise as a *de novo* condensation. Jarošová (1974) described tarsal development in *Xenopus laevis* and *Discoglossus pictus* and established that all distal tarsal elements are present—except Distal Tarsale 5 and Centrale 4 (which would correspond to Distal Tarsale 4) that are incorporated into the fibulare end. Thus, both condensations distal to the fibulare observed in *P. platensis* would correspond to Distal Tarsalia 4 and 5 described by Jarošová (1974).

5. For most anurans, limb ossification takes places in the diaphyses during larval development, whereas epiphyses, carpal, and tarsal ossification/calcification occurs during postmetamorphic growth and ends in large size adults. In *P. platensis* the end of metamorphosis involves the full ossification of the postaxial skeleton, including epiphyses, the carpus and the tarsus. This situation is rare among anurans. Hinchliffe and Johnson (1983) mentioned the absence of endochondral ossification/calcification in the epiphyseal cartilages of amphibians and a pattern of ossification in which the calcification of the diaphyses is continuous with that of the epiphyses, which would be characteristic of anurans. As complete ossification of long bones in anurans, as well as carpal and tarsal elements, takes place in specimens that reach adult size, we could hypothesize the observed pattern of ossification in *P. platensis* precludes an evident postmetamorphic growth of the postcranial skeleton.

6. As was described by Manzano et al. (2007) the intercalary elements of species of *Pseudis* are different from all Hyloides in: a) shape and function, in being cylindrical with plane articular surfaces that allow limited movements; and b) histological composition, as they are formed by hyaline cartilage. Both shape and histological structure suggest that the intercalary of *Pseudis* are peramorphic features. The onset of differentiation of the intercalary element is the same for *P. platensis* and other hylids (such as *Phyllomedusa sauvagii* and *Scinax fuscovarius*; Fig. 6) and it occurs after the complete differentiation of phalanges in manus and pes (Stages 39 and 40). Meanwhile, the development of the intercalary in *P. platensis* occurs quickly and the hyaline cartilage is well differentiated before metamorphosis; the other species show a slower pattern in which numerous fibroblasts define the biconcave disc that gradually proceeds with the arrangement of fibers and differentiation of chondroblasts that occur during metamorphosis. The resulting embryonic cartilage remains during the whole ontogeny of Hyloides. It may be mineralized in some species but never acquires the proteoglycan matrix typical of the hyaline cartilage of *P. platensis* and Ranoides (Manzano et al., 2007). In respect to Hyloides, the intercalary elements of *P. platensis* show an accelerated developmental rate that defines early its elongated shape and the proteoglycan matrix of hyaline cartilage.

To conclude, the study of skeletal development during metamorphosis of *P. platensis* has revealed that this species shows an unusual pattern of development, because at the end of metamorphosis the skull, the hyoid apparatus, and the postcranial skeleton are completely formed and fully ossified. Some skull elements, such as palatines, sphenethmoid, pars externa plectri, and tympanic annulus that are characteristic of juvenile stages for most anurans are present before the tail is completely lost. The postcranial skeleton also displays an intensive ossification in the axial axis, synostosis among girdles bones, and ossification/calcification of epiphyses and mesopodial limb elements. Our findings indicate that the end of metamorphosis in *P. platensis* is the age in which the skeleton stops development and growth, and suggest that this aspect of somatic development is predisplaced and happens during the long metamorphosis of this species.

Furthermore, as metamorphosis in *P. platensis* exhibits dissociated events in the development of the sound conducting apparatus, we present new evidence to interpret the origin of the pars externa plectri from the palatoquadrate cartilage or suggest it is a morphological novelty.

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