Comparative description and ossification patterns of *Dendropsophus labialis* (Peters, 1863) and *Scinax ruber* (Laurenti, 1758)(Anura: Hylidae) (#20437)

Second revision

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Comparative description and ossification patterns of *Dendropsophus labialis* (Peters, 1863) and *Scinax ruber* (Laurenti, 1758)(Anura: Hylidae)

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Although comparative studies of anuran ontogeny have provided new data on heterochrony in the life cycles of frogs, most of them have not included ossification sequences of Neotropical frogs. Using different staining techniques, we describe the cranial and poscranial elements development in two hylid species, *Scinax ruber* and *Dendropsophus labialis*, providing new data for more comprehensive ontogenetic studies in Colombian species. We examined specimens from Gosner stages 25 to 45. We found differences in the infrarostral and suprarostral cartilages, optic foramen, planum ethmoidale, and the gill apparatus. In the ossification sequence, the first elements to ossify were the transverse process of spinal column and atlas in both species, and the parasphenoid in the skull. These two species showed the suprascapular process as have been described in other Hylids species cleared and stained until now. New descriptions of skeletal development and ossification sequences of larval stages of these two species, mainly data concerning the postcranium, contribute with useful information for analysis of sequential heterochrony, because although the hylids are widely known, there are few works (15 of 700 species) about ossification sequence that include the whole skeleton.

Comparative description and ossification patterns of *Dendropsophus labialis* (Peters, 1863) and *Scinax ruber* (Laurenti, 1758) (Anura: Hylidae)

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Short Title: Skeletal development of two hylids

Abstract Although comparative studies of anuran ontogeny have provided new data on heterochrony in the life cycles of frogs, most of them have not included ossification sequences~~ of frogs~~. Using differential staining techniques, we observe and describe differences and similarities of cranial and postcranial development in two hylid species, *Scinax ruber* (Scinaxinae) and *Dendropsophus labialis* (Hylinae), providing new data of ontogenetic studies in Colombian species. We examined tadpoles ranging from Gosner Stages 25 to 45. We found differences between the species in the infrarostral and suprarostral cartilages, optic foramen, planum ethmoidale, and gill apparatus. In both species, the first elements to ossify were the atlas and transverse processes of vertebral column~~,~~ and the parasphenoid. Both species exhibited suprascapular processes as described in other ~~H~~ylids. Although the hylids comprise a large group (over 700 species), postcranial ossification sequence is only known for 15 species. Therefore, the descriptions of the skeletal development and ossification sequences provided herein ~~should~~ be useful for future analyses of heterochrony in the group.

Introduction

Comparative morphological descriptions have provided useful systematic characters since the 1960 (e.g. Cannatella 1999; Duellman et al. 2016). However, most studies of frog morphological characters focus on adults (Faivovich 2002; Faivovich et al. 2005; Maglia et al. 2007; Wiens et al. 2010; Pyron and Wiens 2011; Yıldırım and Kaya 2014; Duellman et al. 2016), and tadpoles ~~are~~ often overlooked (Alcalde et al. 2011). Of those comparative studies that examine tadpoles, most consider external morphological characters and~~,~~ skeletal characters are often neglected (Fabrezi and Lavilla 1992; Faivovich 2002; Maglia et al. 2007; Hoyos et al. 2012; Yıldırım and Kaya 2014). When skeletal features are considered, the chondrocranium is most often described, ~~and~~ the postcranium is frequently ignored (e.g. Orton 1953; Starrett 1973; Wassersug 1980; Wassersug and Heyer 1988; Haas 2003). However, as with other groups, relatively few detailed comparative morphological studies of hylid tadpole skeletal development have been completed. Given the ~~size~~ and recent taxonomic re-arrangements of the hylids (Duellman et al. 2016; Jungfer 2017) is important to amass as much comparative information about the group as possible. Thus, there continues to be a pressing need to conduct comprehensive comparative studies of hylids developmental morphology.

Interspecific variations in morphology help to clarify taxonomic groups within in the Hylidae. The family is predominantly distributed across the Neotropical region (Frost 2018; Duellman et al. 2016) and comprises 706 species ~~subdivided~~ into seven subfamilies: Acridinae, Cophomantinae, Dendropsophinae, Hylinae, Lophyophylinae, Pseudinae, and Scinaxinae (Faivovich et al. 2005; Wiens et al. 2010; Duellman et al. 2016; Frost 2018). Ossification sequences are known for only 15 species, and only eight of those include the postcranial skeleton: *Acris blanchardi* (Havens 2010: Maglia et al. 2007), *Boana lanciformis* (former *Hyla lanciformis,* De Sá 1988), *Boana pulchella* (former *Hypsiboas pulchellus* Hoyos et al. 2012), *Dryophytes chrysoscelis* (former *Hyla chrysoscelis,* Sherman and Maglia 2014), *Dryophytes versicolor* (former *Hyla chrysoscelis,* Sheil et al. 2014), *Hyla orientalis* (Yıldırım and Kaya 2014), *Osteopilus septentrionalis* (Sheil et al. 2014), *Pseudacris crucifer* (Havens 2010).

Because identifying variations in developmental morphology and ossification sequence can lead to informative phylogenetic characters (Weisbecker and Mitgutsch 2010; Harrington et al. 2013), ~~herein~~ we provide a detailed anatomical comparisons of the cranial and postcranial development (including the sequence of onset of ossification) between two species of Andean hylids, *Dendropsophus labialis* and *Scinax ruber*.

Materials and methods

We cleared and double stained for bone and cartilage (Dingerkus and Uhler 1977). We ~~modificed~~ to the protocol: 1, proportion of ethanol (SIGMA Ref. 459836-2L) and acetic acid (SIGMA Ref. K36101663 620) was changed to 70:30; 2, The Alcian blue (SIGMA Ref. A5268-25G) was increased to 75 mg, which was dissolved in ethanol and acetic acid; and 3, staining ~~time~~ of this last solution was increased to 72 hours. The tadpoles and metamorphs ~~number~~ of *Dendropsophus labialis* was N= 32, and *Scinax ruber* was N=114. The number in each series corresponds to the availability of specimens stored at the Museo de Historia Natural “Lorenzo Uribe” at the Universidad Javeriana (MUJ) and the Instituto de Ciencias Naturales at the Universidad Nacional in Bogotá – Colombia (ICN). The larval stages of *D. labialis* were collected from the Municipio Tenjo, Cundinamarca Departament, 3200 m (MUJ 9250). The larval stages of *S. ruber* were collected from the Mun. Neiva, Huila Dep., 570 m; Mun. Granada, Meta Dep., 470 m (MUJ 3727, MUJ 6178, ICN 46015-46017). Tadpoles and metamorphs were staged according to Gosner~~’s~~ (1960).

Observations and photographs were made with a stereomicroscope (Advanced optical), a camera (Infinity 1 Lumenera Corporation) with white LED light and Image Pro Insight program (version 8.0.3). The drawings were made using a digitizing tablet (Wacom Bamboo Connect pen) and edited using Adobe Illustrator 5. Anatomical nomenclature for tadpoles follows Parker 1876; Higgins 1921; Jolie 1962; Roček 1981; Duellman and Trueb 1986; Haas 1995; Haas 1997; Hall and Larsen 1998; Maglia and Púgener 1998; Cannatella 1999; Haas 1999; Sheil and Alamillo 2005; Púgener and Maglia 2007; Bowatte and Meegaskumbura 2011; Hoyos et al. 2012; adult nomenclature is based on Avilán and Hoyos (2006), using the Latin names given by the ICVAN (1973), and taking into account a Nomina Anatomica Batrachologica does not exit.

The ossification sequence was ~~constructed with~~ the first appearance of ~~bone~~. We refer to metamorphic climax (MC) *sensu* Banbury and Maglia (2006) as the Gosner stages at which major modifications and fundamental structural changes occur, resulting in the loss of most of the larval characters. We also used the term "rank" to refer to the ordinal number within an ossification sequence at which an element begins to ossify. We ~~decide on~~ the first time any specimen at that stage showed stain. If two or more elements begin ossifying at the same time Gosner stage, they were assigned the same rank (i.e. a tie) as per Nunn and Smith (1998).

Results

Skeletal development and sequence of onset of ossification of the cranial and postcranial elements of *Dendropsophus labialis* and *Scinax ruber* are showe~~d~~ in Table 1 and 2. A number of specimens in our series of *Scinax ruber* did not show clear staining, thus we increased our sample size. Most of the poorly stained specimens were between Stages 26 and 35) after Stage 35 specimens stained more clearly.

Chondrocranium

We observed similar changes in the shape, size, and modification of structures in the development of chondrocranium in the two species. The elements of the skeleton were compared according to the ~~beginning~~ of ~~the~~ ossification and not ~~in~~ a specific state, because in the two study species this occurred in different Gosner ~~states~~ (Table 3). The overall width of the chondrocranium in *Dendropsophus labialis* and *Scinax ruber* is *roughly* 80-90% of this total length (Fig 1). The chondrocranium in *D. labialis* is wider (dorsal view) and lower (lateral view) than *S. ruber* (Fig 1A, 1B, 1C). Basicranial fenestrae did not differentiate with Alcian Blue in either species. We perceived a stronger blue coloration in *D. labialis*, and the jugular, prootic, and oculomotor foramen were clearly ~~differentiated~~, whereas in *S. ruber* we could not see the oculomotor foramen.

The cartilaginous region of the taenia tecti medialis and tectum sinoticum both represent a quarter of the basis cranii, extending from the frontoparietal fontanelle in both species. The tectum nasi roofs the nasal region, and the ethmoid plate forms the floor. The tectum nasi is separated from the orbit by a wall, the lamina orbitonasalis (=planum antorbitale *sensu* Cannatella 1999). Because these regions are weakly chondrified, the lamina orbitonasalis is not observable in the tadpole stages, and the nasal capsules become visible ~~on~~ after metamorphic climax (Stage 42 and beyond). The taenia tecti marginalis is evident and clearly differentiated by GS37 in *D. labialis* and by GS35 in *S. ruber*. In neither species did we observe a frontoparietal fenestra, nor was a taenia tecti transversalis visible on the edge of the frontoparietal fontanelle (Fig. 1A).

Suprarostral cartilage. In both species, the suprarostral cartilage is composed of a discontinuous cartilaginous plate divided into a corpus suprarostralis and a pars alaris; posterolaterally we observed a distal syndesmotic junction between the corpus and the ala. The ala has three processes: two rounded anterolateral processes that join syndesmotically with the cornu trabecula, and one process posterolaterally (Fig. 1C). Fenestrations were not observed in the suprarostral cartilage nor, in the adrostral cartilage near the processus posterodorsalis (=processus dorsalis posterior, *sensu* Bowatte and Meegaskumbura 2011). In *D. labialis* the corpus suprarostral is curved, while in *S. ruber* it is straighter and wider distally, articulating proximally with the cornu trabecula (trabecular horn, *sensu* Cannatella 1999). The cornua trabecula are approximately 35% of the total length of chondrocranium (lateral view) in both species; they are shorter and narrower in *D. labialis* than in *S. ruber*. The cornua trabeculae articulate anteriorly with the corpus rostrale and laterally with the pars alaris of the suprarostral cartilage.

Cartilago Meckeli. The cartilago Meckeli (= Meckel's cartilage, *sensu* Cannatella, 1999) has three processes: the retroarticular (short and blunt), the dorsomedial, and the ventromedial. These processes articulate with the infrarostral cartilage (commissura intramandibularis, *sensu* Cannatella, 1999) which is composed of two syndesmotically, joined flat plates, and the processus muscularis quadrati; the shape of the processus dorsomedialis and the processus ventromedialis are the same in both species. The palatoquadrate cartilage and the commissura quadratocranialis are joined anteriorly to the base cranii. Laterally, the palatoquadrate cartilage forms the arcus subocularis. The process muscularis quadrati is joined to the processus antorbitalis (= pars plana *sensu* Parker 1876; = lamina externa *sensu* Higgins 1921; = processus antorbitalis *sensu* Roček 1981; = triangular plane *sensu* Hall and Larsen 1998 = cartilaginous planum triangulare *sensu* Púgener and Maglia 2007) anterolaterally, projecting above the cornu trabecula. The processus hyoquadrati of the palatoquadrate cartilage articulates ventrally with the ceratohyalia of the hyobranchial apparatus (Fig. 1D).

Otic capsule. This structure is longer and higher than wide, occupying about a fifth of the total length of the skull. The crista parotica exhibits a more pronounced lateral projection in *D. labialis* than in *S. ruber*. The crista parotica is laterally developed, forming a small processus posterolateralis (= processus lateralis posterior *sensu* Bowatte and Meegaskumbura 2011) and a small processus anterolateralis (more developed in *D. labialis*). The processus anterolateralis projects vertically, descending obliquely and overlapping the ventral posterolateral margin of the palatoquadrate cartilage. The otic capsule is perforated by the fenestra ovalis, which occupies about 20% of the otic capsule.

Hyobranchial apparatus. The large ceratohyal has a processus anterioris hyalis, a processus posterioris hyalis, and a processus anterolateralis hyalis. The first two processes are longer than the third, which extends to meet the transverse crease of the processus lateralis hyalis.

The basihyal plate is oval and extends proximally to the copula anterior (= Basibranchial I *sensu* Duellman and Trueb 1986; = basihyale *sensu* Haas 1995 and Haas 1997; = Copula I *sensu* Maglia and Púgener 1998; Sheil and Alamillo 2005) in *D. labialis*, but is absent in *S. ruber*. The basibranchial plate is semi-oval and located between the two hypobranchial plates (= planum hypobranchiale *sensu* Haas 1999; = *plate hyoid sensu* Maglia and Púgener 1998; = *hyobranchial plate sensu* Sheil and Alamillo 2005) a branchial bridge is present in both species, being wider in *S. ruber* than in *D. labialis*. The junction between each ceratobranchium and the planum hypobranchiale is syndesmotic. The ceratobranchia are united posteriorly by the commissura terminalis and bear three spicules anteriorly (Fig. 1D).

The chondrocranial morphology and hyobranchial apparatus is generally similar between the species examined here~~in~~ and those previously studied ~~but~~ we did identify several differences between ~~in~~: 1) the shape of the suprarostral, 2) the size and width of infrarostral cartilages, 3) the length of processus articularis, 4) the thickness of palatoquadrate, 5) the size of optic foramen, 6) the presence of an operculum and processus posterolateralis of the otic capsule, 7) the thickness of the processus muscularis quadrati, 8) the attachment of the ascending process to the braincase, 9) the thickness of the planum ethmoidale, 10) the development of the branchial apparatus,11) the presence of Copula I, and 12) the type of junction between the ceratobranchia and planum hypobranchiale (Fig. 1 and 2). These differences likely represent species-specific differences ~~among~~ the taxa examined.

Appendicular skeleton

Shoulder girdle. The pectoral girdle is arciferal in both species. The earliest ossification of clavicle, coracoid, and scapula appears at GS36 (Fig. 3A). The clavicle and the cleithrum are distinct, and an epicoracoid cartilage is prominent between the clavicle and the coracoid. The epicoracoids are not mineralized. In *D. labialis* the omosternum is elongated and the sternum has two projections; the omosternum and the sternum are oval in *S. ruber*. The clavicle articulates with the coracoid, which is ossified in *D. labialis* at GS41 and in *S. ruber* at GS46. The sternum is formed by the epicoracoid and the mesosternum, which joins the medial junction of the epicoracoids (Fig. 2B).

Pelvic girdle. In both species, the primordium of the ilium appears at GS34 and is fully developed by GS41. The ilium begins to ossify by GS41 *D. labialis* and by GS39/40 in *S. ruber*, and articulates anteriorly with the ventral surface of the lateral margin of the sacral diapophyses by GS42. The iliac crest appears dorsally prominent; the primordia of the pubis and the ischium appear at GS36, and are synchondrotically fused by GS38 in both species. The sacral diapophyses is wider in *D. labialis* that in *S. ruber*. The pubis is completely fused by GS40. The pelvic girdle is completely ossified with the halves fused at the midline, extending anterodorsally forming an angle of 55º with the head of the femur by GS45 (Fig. 3).

Forelimb and hindlimb. The first cartilaginous elements of the forelimbs (radius, ulna, and humerus) appear at GS32, and those of the hindlimbs at GS33 (femur, tibia, and fibula). The tibia and fibula are fused in *D. labialis* by GS41 and in *S. ruber* by GS38. We observed ossification of the radius and ulna in *D. labialis* (GS41) and *S. ruber* (post metamorphic)~~; these~~ ~~elements~~ are fused in both species. Primordia of the four carpal and five tarsal elements appear by GS33 and complete development by GS41. The phalangeal carpal formula is 3-3-4-4 and the phalangeal tarsal formula is 3-3-4-5-4 in both species. Metacarpals are curved and phalanges are cylindrical, having a conical shape at the tip of the terminal phalanges. Digits IV (manus and pes) and V (pes) begin to ossify by GS42 in *D. labialis*, although all phalanges are ossified at GS45 in both species (Fig. 3). The carpal were cartilaginous in all specimens and stages examined, and the distal tarsals were cartilaginous in *S. ruber*. The relative size of carpal elements is 3 < 4 < 2 < 1< prehallux and the tarsal elements is 4 < 5 < 3 < 2 < 1 < prepollex. Sesamoids are absent from GS25 to GS45. Figure 3A shows the limb elements (central, fibulare, radiale, tibiale, ulnare, and intermedium) at Stage 45.

Axial skeleton

The vertebral column is composed of eight procoelous presacral vertebrae, the sacrum, and the urostyle. The notochord diminishes as the tadpoles grow~~,~~ and is complete resorbed by GS44 in both species (Fig. 4). We found that the axial skeleton was more chondrified in *D. labialis* than in *S. ruber*. The first postcranial skeletal elements to develop in both species were the nine pairs of semicircular cartilaginous primordia of neural arches, includ~~ed~~ eight presacral vertebrae, the sacrum, the urostyle and the hypochord. The sacral diapophyseal primordia are cylindrical. The last postsacral vertebra (first coccygeal or Vertebra X *sensu* Haas 1999) and the second coccygeal vertebra ossify only in *D. labialis* by GS45. Simultaneously to the ossification of presacral vertebrae, there is notochord absorption, coccygeal elements ~~fusion~~ and urostyle formation. The urostyle has a bicondylar articulation with the sacral vertebra and the condyles are widely separated in both species (Fig. 4).

The atlas is concave at its point of articulation with the convex occipital condyles at the base of the skull. Semicircular procoelous (*sensu* Jolie 1962) vertebral centra begin to develop as early as GS31 in *D. labialis* and GS32 in *S. ruber*, increasing the thickness of both the neural arches and the transverse process. The neural arches appear as cartilage at GS33 in both species~~; these are~~ complete at GS34 in *D. labialis* and at GS38 in *S. ruber*. The arches are fused dorsally at the midline at GS38 in *S. ruber* and at GS38 in *D. labialis*. The transverse processes are the first elements to ossify in both species (Tables 1 and 2). Postzygapophyses and prezygapophyses are conspicuous in presacral vertebrae II, III, and IV in both species. Sesamoids are absent from GS25 to GS45.

Ossification sequence

The earliest stage examined in both species was GS25. Ossification in *D. labialis* appears by GS34 and in *S. ruber* by GS35 (Fig. 1A and Fig. 4). Ossification in *D. labialis* begins with the atlas and the transverse processes, whereas in *S. ruber* it begins with the parasphenoid, the Transverse Processes I-VII and Neural Arches I-III.

Metamorphic climax (MC) begins at GS41 in *D. labialis* and GS39-40 in *S. ruber*. We identified seven ranks (I–VII) in *D. labialis* and five ranks (I–V) in *S. ruber* (Tables 1 and 2). Ossified elements were perceptible in *D. labialis* from GS35 to GS45, with 46 ossified elements, and from GS36 to GS43 in *S. ruber*, with 26 ossified elements. Metamorphic climax in *D. labialis* was at GS45 with 14 ossified elements and in *S. ruber* at GS39-40 with seven ossified elements. Of these, the structures in common are the femur, tibia, fibula, humerus, ilium, and radioulna.

Discussion

Despite Colombia housing the second number of hylid species on the planet, few previous studies have considered developmental ossification of Colombian hylids. The family Hylidae has gone through a number of taxonomic re-arrangements, as elucidated by various phylogenetic hypotheses based on molecular, chromosomal, and morphological data from both larvae and adults (Faivovich 2002; Faivovich et al. 2005; Wiens et al. 2010; Pyron and Wiens 2011; Duellman et al. 2016). Data from additional morphological studies of Colombia hylids may help to support or refute these hypotheses.

Previous studies the cranial morphology in hylid tadpoles include *Acris crepitans* (Maglia et al. 2007); *Boana lanciformis* (de Sá 1988; Alcalde and Rosset 2003); *Boana pulchella* (Hoyos et al. 2012); *Boana raniceps* and *Dendropsophus nanus* (former *Hyla raniceps* and *Hyla nana* Fabrezi and Lavilla 1992; Vera Candioti et al. 2004); *Dryophytes versicolor* (former *Hyla versicolor,* Sheil et al.2014); *Hyla orientalis* (Yıldırım and Kaya 2014); *Julianus acuminatus* (former *Scinax acuminatus*, Fabrezi and Lavilla 1992; Faivovich 2002; Alcalde and Rosset 2003; Alcalde et al. 2011), *J. uruguayus, J. aff. pinimus* (former *Scinax uruguayus* and *Scinax aff. pinima*, Alcalde et al. 2011; Rodrigues et al. 2017); *Ololygon aromothyella* and *O. berthae* (former *Scinax berthae*, Rodrigues et al. 2017; Alcalde et al. 2011; Faivovich 2002); *O. skuki* (Rodrigues et al. 2017 *Scinax granulatus* and *S. squalirostris* (Rodrigues et al. 2017 Alcalde and Rosset 2003); *S. boulengeri* (Rodrigues et al. 2017; Vera Candioti 2007); *S. fuscovariatus* (Fabrezi and Vera 1997); *S. nasicus* (Rodrigues et al. 2017; Vera Candioti 2007; Vera

Candioti et al. 2004; Fabrezi and Vera 1997)~~;~~ *S. ruber* (Haas 1996). For a complete overview of the findings of these studies please see Appendix 1.

Several of the differences between the two species examined present interesting avenues for future examination. For example, processus ethmoidalis of the quadrate in *S. ruber* is wide, and it is not clearly distinct from the processus articularis~~,~~ ~~whereas in~~ ~~*D. labialis*~~*,* the processes are easily distinguishable~~,~~ and similar to that described by Alcalde and Rosset (2003), who found similar features in *Boana raniceps* compared with ~~to~~ the *Scinax* group (*S. squalirostris* and *S. granulatus, Scinax ruber* group). The palatoquadrate is similar ~~between the species~~ but the processus ascendens of the palatoquadrate in *D. labialis* is wider than in *S. ruber*, and the distal side of the cornu trabecula extends posteriorly toward the otic capsule~~, ~~the anterior region of the palatoquadrate is distinctively broader in *S. ruber* than in *D. labialis*, in *S. ruber* the dorsomedial process is wider than the ventromedial process in *D. labialis*.

When comparing the decelopment of ~~in~~ *D. labilis* with *D. nanus* (Vera Candioti et al. 2004; Alcalde and Rosset 2003) we found that *D. labialis* can be differentiated by the reduction of the buccopharyngeal and branchial basket structures~~,~~ and ~~the~~ sinus posterior hypobranchialis and ~~the~~ processus quadrato-ethmoidale ~~are missing~~. On the other hand, the information that is available for *Scinax* species (Fabrezi and Lavilla 1992; Haas 1996; Fabrezi and Vera 1997; Faivovich 2002; Alcalde and Rosset 2003; Vera Candioti et al. 2004; Vera and Haas 2007; Alcalde et al. 2011; Rodrigues et al. 2017) shows that there are ~~a great morphological~~ variations that require~~s~~ extending the morphological studies in tadpoles.

Scinax ruber presents alae and corpus of suprarostral cartilage with deeper notches;

the chondrocranium, hyobranchial apparatus, and the suprarostral body joined syndesmotically as found by Vera and Haas (2007) in microphagous tadpoles of *S. nasicus* and *S. boulengeri*. *Dendropsophus labialis* like *Dendropsophus nanus* shows a suprarrostral cartilage with corpus and alae forming a continuous structure which is, evidently, associated with a deviation of the macrophophagous mechanisms described by Alcalde and Rosset (2003).

The lateral development of the crista parotica is more prominent in *S. ruber* than in *D. labialis*. It is possible that some of the variation in the anatomical structures of the otic capsule are functionally related to perception of vocalizations (i.e. same species recognition) in adult stages, but experiments must be conducted to ~~check~~ the relationship of these anatomical structures with hearing physiological functions (Ruggero and Temchin 2002; Boistel et al. 2013).

The chondrification of skull in *S. ruber* is faint when viewed laterally, and foramina are not clearly visualized. By contrast, in *D. labialis*, much more blue coloration was observed. This could be due to the abundant chondrification of these parts or to the early developmental stages of this anatomical area in which it is allowed differentiation of craniopalatine carotid foramina.

Although the sample size for the *Dendropsophus labialis* is very small in comparison with *S. ruber*, *Dendropsophus labialis* exhibited more ossified elements with stronger chondrification and less intraspecific variation, while *S. ruber* showed more intraspecific variation and less overall chondrification in the samples (Fig. 1). *D. labialis* presented uniformly stained (ossified) elements in all individuals (Table 3).

This variation between *S. ruber* and *D. labialis* could be caused by intrinsic factors that determine the timing of development or extrinsic factors affecting osteogenesis (Vera and Ponssa 2014). It may not be a coincidence that *S. ruber* is a generalist species and *D. labialis* is an endemic one (Frost 2018).

Haas (1996) reported that the Ceratohyalia II-IV are fused in *Scinax ruber* and *Megophrys montana nasuta*, characteristics that separate them from other species. ~~Herein, this state was~~ confirmed in *S. ruber* but not in *D. labialis*. The ceratohyal in *D. labialis* has a process on the articular condyle that is not present in *S. ruber*. Alcalde and Rosset (2003) found this process in both *S. granulatus* and *S. squalirostris.* Spicules I-III on the posterior margin of the hypobranchial plate are present in *D. labialis* and *S. ruber*, but Spicule IV is not.

Copula II is present in both species~~; but~~ Copula I is present in *D. labialis* as in *S. squalirostris*, but absent in *S. ruber* as in *S. granulatus, Boana raniceps* (Alcalde and Rosset 2003) and *Tlalocohyla smithii* (Vera and Haas 2004). Although the presence of Copula I is extremely variable in hylids~~,~~ and is shared by all non-hylid (Vera and Haas 2004), a relationship between this structure and the ecological function that it performs (e.g. prey utilization) has not been identified.

Additional characteristics of the developmental morphology of these close species them with other hylids that have been studie~~s~~ previously. For example, the urostyle of *D. labialis* and *S. ruber* forms a bicondylar articulation with the sacral vertebra and the condyles are widely separated. The shoulder girdle of both species present differences in the shape of the omosternum and sternum at GS 45. *Dendropsophus labialis* and *S. ruber* present suprascapular processes in tadpoles and adults similar to those in other hylids~~:~~ *Hypsiboas lanciformis* (De Sá 1988), *Boana pulchella* (Hoyos et al. 2012), *Pseudacris crucifer, Acris blanchardi* (Havens 2010) and *A. crepitans* (Maglia et al. 2007) but is absent in *Scinax catharinae* Clade (Faivovich et al. 2005).

Variations of larval characters between *Scinax* and *Dendropsophus* have been included in several phylogenetic studies (Fabrezi and Vera 1997; Haas 1996; 1999; 2003; Alcalde and Rosset 2003; Vera 2007). In our study, the skeleton shows significant differences between the species *Scinax ruber* and *Dendropsophus labialis*, beginning with fact that elements ossified in *S. ruber* exhibit more intraspecific variability than in *D. labialis* (see Table 3).

Regarding ossification sequence, the first bones ossified in the cranium were the exoccipital, the frontoparietal and the parasphenoid by GS 36. Haas (1999) found that in *S. ruber* this occurre one stage later by GS37. Similar to those that Haas (1999) described for other hylids, the ossification of the vertebrae begins from the centra of the presacral vertebrae and continues ventrally along the notochord, forming osseous rings around the notochord in both species. We found that the ossification of the centra in both species we studied begins ventrally and proceeds dorsally. Haas (1999) recorded the ~~ossification~~ transverse processes of Presacral Vertebrae II-III as the first to ossify, while we found that the timing of ossification of Neural Arches ~~goes~~ from I to IX in *D. labialis* (GS37) and from I to III in *S. ruber* (GS36) (Fig 4).

The detection of more intraspecific variability in *Scinax ruber* than in *Dendropsophus labialis* could also be due to the presence of more intra-generic diversity in the clade ~~*Scinax ruber*~~. Alcalde and Rosset (2003) associated the type of feeding with the development of the lateral anterohial process of the ceratohial, in species with macrophage larvae (*Dendropsophus nanus*), and scraping microphages (*Boana* *pulchellus, Julianus acuminatus* and *S. nasicus*). This may indicate that morphological characteristics of the jaw may be involved in the ~~type of~~ feeding ~~that~~ the tadpoles ~~have~~ and therefore these traits would help to~~identify~~ the species (Appendix 1).

Differences between the ossification sequences of these two species are also evident when examining the ossification ranks and number of ossified bones~~;~~ in particular, *D. labialis* has more ranks in the sequence and more elements that begin ossification prior to metamorphosis. With respect to the postcranium, the number of elements ossified appears earlier in *D. labialis* than in *S. ruber.* Because Gosner stages are based on external characteristics that rely on underlying skeletal change, it is only a relative measure of timing and should not be used as a way to compare ~~between~~ species. Instead, we compared the relative timing of events in the ossification sequence~~,~~ by examining the order of onset of ossification of each element. Nunn and Smith (1998:86) considered “ontogeny may be ordered by age, size, or stage; none of these measures are useful for comparing ontogeny across significantly divergent taxa”.

Table 4 outlines the ossification sequences of different species of the family Hylidae ~~among these species,~~ the number of ranks that include elements of the skull and postcranium vary from one to five. The number of ranks increase when postcranial elements are included. Weisbecker and Mitgutsch (2010), Harrington et al. (2013), and Sheil et al. (2014) used similar ranked ossification sequence data to reconstruct phylogenetic trees of amphibians in the families of Leptodactylidae, Ranidae and Bufonidae. These researchers suggested ~~to use~~ cranium and post-cranium data, relating them to the type of development, and to include sequences of fossils~~ too,~~ as far as possible.

Although the morphology and systematics of amphibians have been extensively studied (Cannatella and Trueb 1988; de Sá and Hillis 1990; Baez and Pugener 2003; Roelants and Bossuyt 2005; Faivovich et al. 2005, Frost et al. 2006; Pyron and Wiens 2011; Duellman et al. 2016), additional comprehensive descriptions of skeletal development and ossification sequences truly understand patterns of heterochrony in the group (Appendix 1).

Some of the biological implications of heterochrony, which are well known in amphibians (Alberch 1985; Reilly et al. 1991) include changes in structures, and changes in the rate of growth of entire organisms (Raff 1996; Smith 2001; 2002; 2003). Some scholars have recognized that heterochrony may work as modules of developmental events with evolutionary implications that can promote or restrict the development of individual morphologies (Wagner 1996).

Studies that have used statistical methods (e.g. Parsimov) to analyze ossification sequences have revealed heterochrony in the timing of onset ossification in some cranial elements such as parasphenoid and prootic in *S. ruber* vs *H. pulchellus* (Hoyos et al. 2012), or the frontoparietal, dentary and maxilla in *D. labialis* vs. *Pseudis platensis* (Fabrezi and Goldberg 2009). In our study, we found that the parasphenoid was the first element to ossify in both *D. labialis* and *S. ruber* and the exoccipital; frontoparietal and prootic were the second elements to ossify in both species.

It is possible that the difference in cartilage formation between the two species examined herein is due to paracrine factors induced in cells that express mesodermal transcription factors involved in the activation of genes specific to cartilage (Gilbert 2000; Kozhemyakina et al. 2015); however, we did not account for these factors.

Additionally, the intraspecific variation in the ossified elements between these species could be linked to specific genes (Raff 1996).

Conclusions

The contribution of ontogenetic data (development and ossification sequences of skeletal structures) ~~herein~~ provides further information to help understand the interactions between ontogeny and phylogeny in morphological and ecological diversity of frog. Ossification sequence data combined with evolutionary hypotheses may shed light on patterns of development to be used in future phylogenetic hypotheses. As Larson et al. (2003) suggested~~:~~ “variation in chondrocranial morphology in larval anurans can be phylogenetically informative, even among closely related taxa”.

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Table 1(on next page)

Ossification sequence of cranial and postcranial elements in *Dendropsophus labialis* (Peters, 1863)

Rank	~~Development~~ stage (Number of specimens ~~Gosner, 1960~~)	Elements ossified	
		Cranium	Postcranium
I	26(3), 27(3), 28(2), 29(2), 31(3), 32(1), 33(1), 34(1)		
	35 (1)		Transverse process I–V
II	36 (2)	Parasphenoid	Transverse process VI–VIII
III	37 (3)	Frontoparietal, exoccipital	Neural arches I–VIII
IV	38 (1)		Hypochord
V	41 (3)		Femur, tibiofibula, humerus, ilium, radioulna, clavicle, pubis, metatarsal III–V, coracoids
VI	42 (3)		Metacarpal IV, urostyle
VII	45 (1)	Mentomeckelian, premaxilla, maxilla, angulosplenial, dentary	Manus IV proximal phalange, Metacarpal III and V, scapula, pedal digit IV proximal phalange, Metacarpal I and II, metatarsal I, prepollex
VIII	46 (1)	Neopalatine, nasal, pterygoid, vomer, septomaxilla, squamosal	

Table 2(on next page)

Ossification sequence of the cranial and postcranial elements in *Scinax ruber* (Laurenti, 1758).

Rank	~~Development~~ stage (Number of specimens ~~Gosner, 1960~~)	Elements ossified: Cranium	Elements ossified: Postcranium
I	26(3), 29(8), 30(7), 31(9), 32(8), 33(9),34(9), 35(14)		
	36 (10)	Parasphenoid	Transverse process I–VII
II	37 (11)		Neural arch I–III
III	38 (4)	Frontoparietal, exoccipital	Transverse process VIII, neural arch IV–VIII
IV	39–40 (3)		Femur, tibiofibula, humerus, ilium, radioulna, scapula, hypochord
	41(4), 42(5)		
V	43 (3)		Ischium
	44(4), 45(2)		
VI	46 (1)	Mentomeckelian, premaxilla, maxilla, angulosplenial, dentary, neopalatine, pterygoid, vomer, septomaxilla, squamosal	

Table 3 (on next page)

Onset of ossification of cranial and poscranial elements of *Dendropsophus labialis* and *Scinax ruber*

Species			Dendropsophus labialis									Scinax ruber									
Gosner Stage (No. Specimens with elements ~~ossified~~)			35 (1)	36 (2)	37 (3)	38 (1)	41 (3)	42 (3)	43 (1)	45 (1)	46 (1)	36 (6)	37 (9)	38 (3)	39/40 (3)	41 (4)	42 (5)	43 (3)	44 (4)	45 (1)	46 (1)
Cranium	Paraesfenoids			1	1	1	3	3	1	1	1	3	5	2	3	3	3	3	3	1	1
	Exoccipital			1	1	1	3	3	1	1	1			3	3	3	4	3	3	1	1
	Frontoparietal				1		2	3	1	1	1			2	2	2	1	3	3	1	1
Poscranium	Transverse Process	I			1							6	9	3	3	3	4	3	3	1	1
		II	1	1	1	1	3	3	1	1	1	5	6	3	3	3	4	3	3	1	1
		III	1	1	1	1	3	3	1	1	1	5	6	3	3	3	4	3	3	1	1
		IV	1	1	1	1	3	3	1	1	1	5	6	3	3	3	4	3	3	1	1
		V	1	1	1	1	3	3	1	1	1	3	6	3	3	3	4	3	3	1	1
		VI	1	1	1	1	3	3	1	1	1	3	6	3	3	3	4	3	2	1	1
		VII	1	1	1	1	3	3	1	1	1	1	4	2	3	3	3	3	2	1	1
		VIII		1	1	1	3	3	1	1	1	1	2	2	3	3	2	3	1		
		IX			1									1	3	1	1	3			
	Neural arch	I		1	1	1	3	3	1	1			3	3	3	3	4	3	3	1	1
		II			1	1	3	3	1	1			3	3	3	3	3	3	3	1	1
		III			1								2	3	3	3	3	3	3	1	1
		IV			1									3	3	3	3	3	2	1	1
		V			1									2	3	3	3	3	2		
		VI			1									2	3	3	1	3			
		VII			1									1	3	2		3			
		VIII			1									1	3	1		3			
		IX																			
	Man	Metacarpal I			1					1											

		Metacarpal II			1					1											
		Metacarpal III			1					2	1										
		Metacarpal IV			1			1		2	1										
		Metacarpal V			1					2	1										
		Metatarsal distal tarsal IV			1					2	1										
		Metatarsal distal tarsal IV			1					1											
		Prepollex			1					1											
		Metatarsal I			1					1											
		Metatarsal II			1			1		1	1										
		Metatarsal III			1		1	2		1	1										
		Metatarsal IV			1		1	2		1	1										
	Pes	**Metatarsal V**			1		1	2		1	1										
	Atlas		1	1	1	1	3	3	1	1	1			3	3	3	4	3	3	1	1
	Femur				1		2	3	1	1	1				3	1		3	1		
	Fibula				1		2	3	1	1	1										
	Tibia				1		2	3	1	1	1										
	Tibiofibula				1		2	3	1	1	1				3	1	1	3	1	1	
	Scapula				1		2	2		1	1				3	1		3			
	Suprascapula				1		2	2		1	1										

	Clavicle			1		2	2		1	1				3	1		3		1	
	Coracoids			1		2	2		1	1										
	Humerus			1		2	3	1	1	1				1	1		3			
	Ulna			1																
	Radioulna			1		2	3	1	1	1				3	3		3			

Table 4(on next page)

Ossification sequences of different species of the family Hylidae including postcranial elements.

Subfamily	Specie	Element	No. ranks	Ossification sequence	References
Acridinae	*Acris blanchardi*	C	9	ps [ex, fp, po] [pm, sm]ma, ns, vo [an, de][me, pt, qj, qu, sq] sp	Havens, 2010
		C, P	11	ps, ve [ex, fe, fp, po][fi, hu, mt, pf, ra, sc, ul, tf, ti][cl, co, ct, il, mc, ph][is, pm, sm]ma, ns, vo, [an, de][me, pt, qj, qu, sq] sp	
	Hyliola regilla	C	6	ps, fp [ex, po] pv [ma, ns, pm, sm, sq] [ag, de, pt][cm, me, pa, qj, sp]	Gaudin, 1973
	Pseudacris crucifer	C	8	ps [ex, fp] [pf, po] [ma, pm, sm] ns [an, vo] [me, pt, qj, qu, sq]sp	Havens, 2010
		C, P	13	ps ,ve, fe [hu, il, ra, su, ul] [ct, ex, fp, fr, sc, tf, tl] [cl, co, mt, pf, po] [mc, ph] is [ma, pm, sm] ns [an, vo] [me, pt, qj, qu, sq] sp	
	Pseudacris triseriata	C	4	[ex, fp, pm] [de, ma, ns, pt, qj, sq, vo][m, po][cm, ha, pa, ps, sp]	Stokely & List 1954
Dendropsophinae	*Dendropsophus labialis*	C	3	ps [ex, fp, po] [an, de, ma, me, np, pm, sm, sq, vo]	**This study**
		C, P	8	[ve] [ps] [ex, fp, po] [cl, co, fe, hu, il, mt, ru, tf] [mc, sc][an, de, ma, me, np, pm, sm, sq, vo]	
Cophomantinae	*Boana lanciformis*	C	8	fp, ps, ex, po [pm, sm] [ns, ma][an, de, sq][sp, me, qj, vo, pa, pt]	De Sá, 1988
		C, P	9	fp [ps, ve] ex [fr, cl, co, ct, fe, hu, mc, mt, po, sc, tf, tl] pf [pm, sm] [ns, ma][an, de, is, sq][ap, me, pa, ph, pt, qj, sp, vo]	
	Boana pulchella	C	2	[ex, fp, ps] [an, de, ma, pm, po, sq]	Hoyos *et al.*, 2012
		C, P	4	[ex, fp, ps, ve][fe, hu, il, ru, sc, tf][cl, co, ct, hy, mc, mt, pf, ph][an, de, ma, pm, po, sq]	
Hylinae	*Dryophytes chrysoscelis*	C	8	ps [ex][fe][fp][sm, pm, po][ma][de, ns, an, sq][vo]	Sherman and Maglia, 2014

		C, P	10	ps [ex, na, cn][fe][sc, cl, co, hu, ra, ul, il, ti, fi, tb,mc, mt][ph, pf][fp][sm, pm, po][ma, is][de, ns, an, sq][vo]	
	Dryophytes versicolor	C	6	ps [ex, fp] [ma, pm, po] [an, de, sq] [pa, pt, qj]	Sheil *et al.*, 2014
		C, P	7	ps [cl, co, fe, fi, fr, hu, il, na, ra, sc, ti, tl, ul] [ex, fp, mc, ph, pf, ve] [ma, pm, po] [an, de, sq] [ns, me] [pa, pt, qj]	
	Hyla orientalis	C	5	ps [ex, po] fp [ns, sm] [an, de, ma, pm, pt, sq, vo]	Yıldırım and Kaya, 2014
		C, P	6	ps [ex, hu, na, po, ve][ct, fe, fr, il, mc, tf, tl, ru][fp, cl, co, mt, pf, ph][ns, sm][an, de, is, ma, pm, pt, sq, pu, vo] qj	
	Smilisca baudinii	C	7	fp [ex, ps, sm] [ag, de, ma, pm, sq] [ns, pt, qj] [me, pa, pv][cm, sp] po	Trueb, 1966
				[ex, fp, ps, sm][ma, pm, sq][ns, pt][pa, qj, vo][cm, et] so, po	Gaudin, 1973
	Triprion petasatus	C	6	fp, ns [an, de, ex, ma, me, pa, pm, ps, pt, qj, sm, sq][cm, pv] sp, po	Trueb, 1970
Lophyohylinae	*Osteopilus septentrionalis*	C	3	fp, sm [ag, cm, de, et, ex, ma, me, ns, pa, pm, po, ps, pt, qj, sq, vo]	Trueb, 1966
			7	fp, sm [an, de, ex, ma, ns, pm, ps, pt, pv, sq] pa, qj [me, po, sp] cm	Trueb, 1970
			5	[fp, sm][ag, de, ex, ns, ma, pm, ps, pt, pv, sq] pa, qj [po, sp]	Gaudin, 1973
		C, P	13	ps, ve, fe [hu, il, ra, su, ul] [ct, ex, fp, fr, sc, tf, tl] [cl, co, mt, pf, po] [mc, ph] is [ma, pm, sm] ns [an, vo] [me, pt, qj, qu, sq] sp	Sheil et al., 2014
Pseudinae	*Pseudis platensis*	C	5	[fp, ps] [ex, po] [ns, pm, sq] ma [de, pt, vo]	Fabrezi and Goldberg, 2009
Scinaxinae	*Scinax ruber*	C	3	ps, [fp, po] [an, de, ma, me, np, pc, pm, sm, sq, vo]	**This study**
		C, P	7	ps, ve [ex, fp, po][fe, hu, il, ru, sc, tf, tl] is [an, de, ma, me, pm, pc] [np, sm, sq, vo]	

C, cranium; P, poscranium

ag, angular; an, angulosplenial; ap, plectral apparatus; cl, clavicle; cu, columella; co, coracoid; ct, cleithrum; de, dentary; et, ethmoid; ex, exoccipital; fe, femur; fi, fibula; fp, frontoparietalis; fr, fibulare; ha, hyoid apparatus; hu, humerus; hy, hypochord; il, ilium; is, ischium; ma, maxilla; mc, metacarpals; me, mentomeckelian; mt, metatarsals; na, neural arches; nc, neural center; np, neopalatine; ns, nasal; pa, palatine; pc, coronoid process; pr, presacral vertebrae; pf, phalanges of feet; ph, phalanges of manus; pm, premaxilla; po, prootic; ps, parasphenoid; pt, pterygoid; pv, pre vomer; qj, quadratojugal; qu, quadrate; ra, radius; ru, radioulna; sc, scapula; sm, septomaxilla; sp, sphenethmoid; sq, squamosal; su, suprascapula; ti, tibia; tf, tibiofibula; tl, tibiale; tp, transverse process; ul, ulna; ve, vertebra (including na, nc, pr, tp); vo, vomer.

C = Cranial elements; P = Postcranial elements.

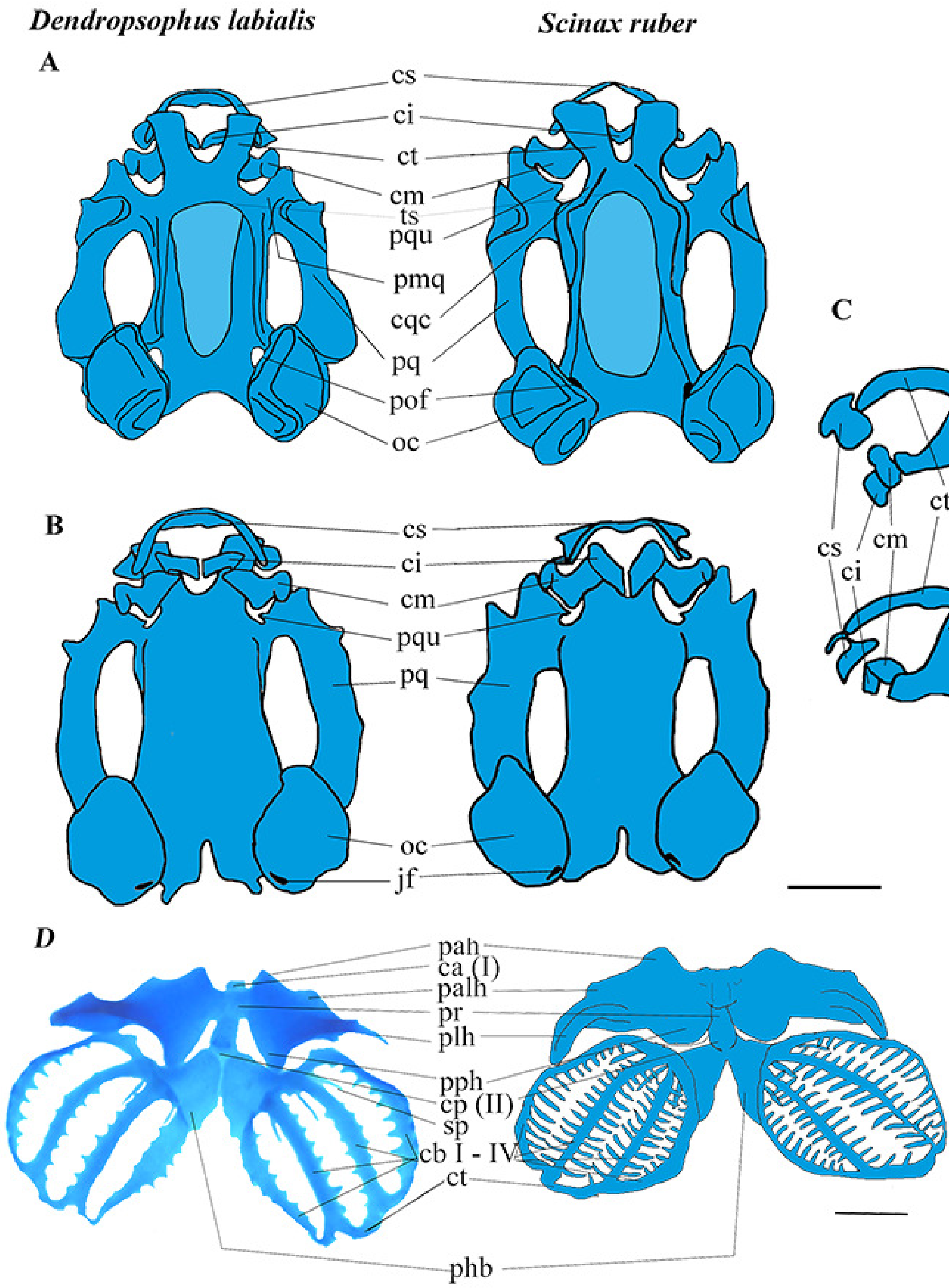
[Rank = absolute time of ossification of various structures simultaneously]

Figure 1

Larval chondrocranium of *Dendropsophus labialis* (GS 34 - MUJ 9250) and *Scinax ruber* (GS 34 - MUJ 6178)

A. Dorsal view, B. Ventral view, C. Lateral view, D. Ventral views of hyobranchial apparatus in *Dendropsophus labialis* (GS36 - MUJ 9250) and *Scinax ruber* (GS36 - MUJ 3727).

Scale 1 mm. Chondrocranium: a, alae suprarostralis; ci, cartilago infrarostralis; cm, cartilago Meckeli; cqc, commissura quadratocranialis; cs, suprarostral cartilage; ct, cornu trabeculae; fo, fenestra ovalis; jf, jugular foramen; pal, processus anterolateralis; pmq, processus muscularis quadrati; pof, prootic foramen; pq, palatoquadrate; oc, otic capsule; of, oculomotor foramen; opf, optic foramen, ts, tectum sinoticum. Hyobranchial apparatus: ca (I), copula anterioris; cb I–IV; ceratobranchialis I–IV; cp (II), copula posterioris; ct; commisura terminalis; pah, processus anterioris hyalis; palh, processus anteriolateralis hyalis; phb, planum hypobranchiale; plh, processus lateralis hyalis; pph, processus posterioris hyalis; pr, pars reuniens; sp, spicula. Blue: cartilage, light blue: fontanella.



Dendropsophus labialis
Scinax ruber
A
cs
ci
ct
cm
ts
pqu
pmq
cqc
pq
pof
oc
B
cs
ci
cm
pqu
pq
oc
jf
C
D. labialis
cs
ci
cm
ct
pmq
opf
of
oc
pof
fo
S. ruber
D
pah
ca (I)
palh
pr
plh
pph
cp (II)
sp
cb I - IV
ct
phb

Figure 2

Appendicular skeleton of *Dendropsophus labialis* (GS45 – MUJ497) and *Scinax ruber* (GS45 – MUJ6018).

A. Scapula, B. Pectoral girdle, C. Ventral view pelvic girdle. Scale 1 mm. c, clavicle; co, coracoid; e, epicoracoid; ps, processus suprascapularis; o, omosternum; st, sternum; il, ilium; is, ischium; pu, pubis Red, ossified; blue, condrificated.

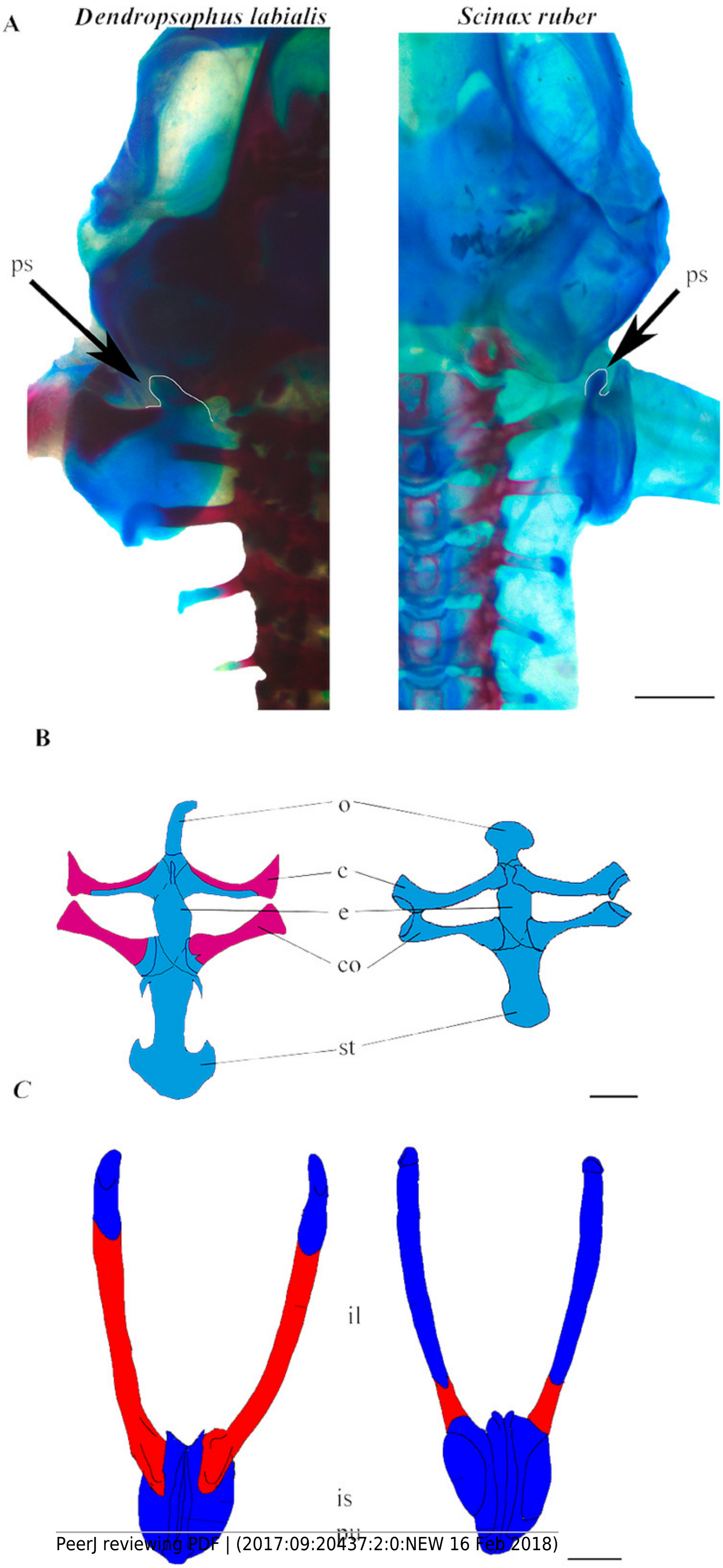

A
Dendropsophus labialis
Scinax ruber
ps
ps
B
o
c
e
co
st
C
il
is

Figure 3

Dorsal view of manus and pes of *Dendropsophus labialis* (GS45 – MUJ497) and *Scinax ruber* (GS45 – MUJ6018).

A. Manus, B. Pes. Scale 1 mm. ce, centrale; fi, fibulare; mc, metacarpal, mt, metatarsus; ph, prehallux; pr, prepollex; rd; radiale; rad, radioulna; ul, ulnare and intermedium; ti, tibiale. I–V, phalanges. Red, ossified; blue, condrificated.

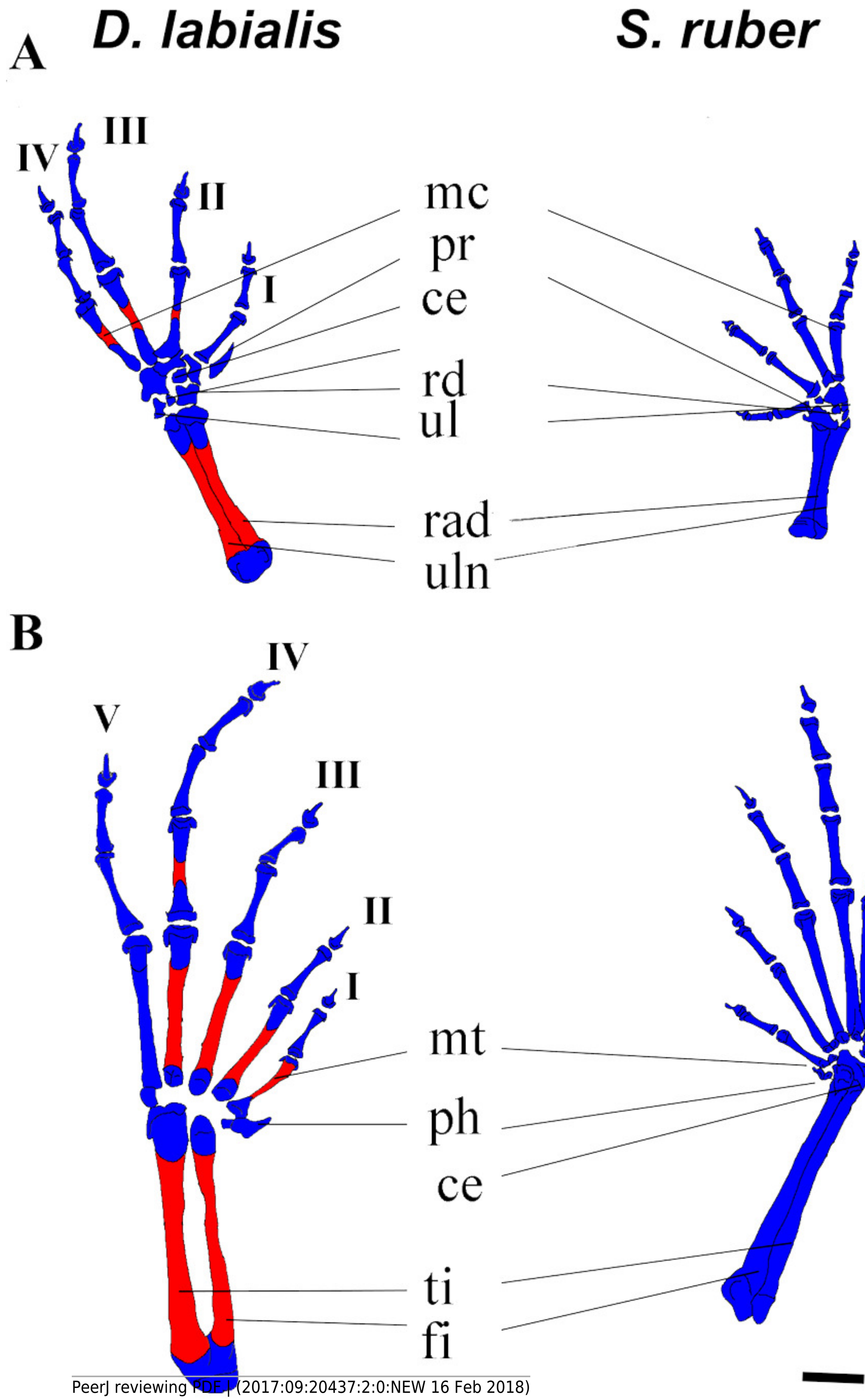

A
D. labialis
S. ruber
IV
III
II
I
mc
pr
ce
rd
ul
rad
uln
B
IV
V
III
II
I
mt
ph
ce
ti
fi

Figure 4

Ventral view of ossification development in vertebral column of *Dendropsophus labialis* and *Scinax ruber* at GS 26-45.

Scale 1 mm. h, hypochord; a, Atlas; np, neural process; d, diapophysis; sd, sacral diapophysis; u, urostyle Red, ossified; blue, condrificated.