Zoologischer Anzeiger 267 (2017) 101–110

Contents lists available at ScienceDirect

Zoologischer Anzeiger

journal homepage: www.elsevier.com/locate/jcz

Zoologischer Anzeiger

Delayed osteogenesis and calcification in a large true toad with a comparative survey of the timing of skeletal ossification in anurans

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ARTICLE INFO

Article history:
Received 23 December 2016
Received in revised form 3 March 2017
Accepted 3 March 2017

Keywords:
Anura
Bufonidae
Rhinella arenarum
Skeletogenesis
Ossification sequence
Histology

ABSTRACT

Postembryonic skeletogenesis in anuran amphibians has been widely studied, yet less than one percent of the extant diversity has been covered and relatively few comparative studies exist. Here we document the sequence and timing of ossification of the Common Toad *Rhinella arenarum*, a large true toad (Bufonidae) from South America that is a model organism for varied ongoing research. We study histological sections and cleared-and-stained specimens of an ontogenetic series ranging from early larval stages to juveniles, documenting the ossification sequence of the entire skeleton. To diminish potential environmental biases we also study the skeletogenesis of the frog *Leptodactylus latinasus* (Leptodactylidae) from the same pond and season. We summarize comparative data from numerous anuran species to contextualize our results in a broad phylogenetic context. Histological data shows that skeletal calcification in *R. arenarum* is temporally dissociated from osteoid matrix formation and occurs later than in most other anurans, which is unexpected given its generalized pond-type larva and heavily ossified adult skeleton. At the onset of metamorphosis, exoccipitals, parasphenoid, and frontoparietals are the only ossified skull elements, whereas most of the postcranium has already started ossification. This pattern is rare among anurans but is shared by other bufonids, in which it has been previously linked to rapid development. Our comparative survey, however, suggests that the delayed bufonid pattern is not related to fast-developing larvae but instead might be a distinctive feature of true toads.

1. Introduction

Postembryonic skeletogenesis in anuran amphibians has been intensively studied, with many previous work focusing on osteological descriptions and ossification sequences of particular species (e.g., Haas, 1999; Hall and Larsen, 1998; Maglia, 2003; Maglia and Pugener, 1998; Shearman and Maglia, 2015; Trueb and Hanken, 1992; Vera and Ponssa, 2014; Wild, 1997; Yıldırım and Kaya, 2014). However, less than one percent of the extant anuran species have been analyzed so far and this type of information has seldom been summarized by comparative studies (e.g., Harrington et al., 2013; Trueb, 1985; Weisbecker and Mitgutsch, 2010), and most of the latter have focused on the skull. Although a fairly conserved developmental pattern exists, there is considerable variation among anurans in the sequence of appearance of particular bones, timing of ossification relative to external morphology, and larval period length (Fabrezi, 2011; Harrington et al., 2013). Interestingly, bones that appear late in the ossification sequence of many anurans convergently fail to ossify in other lineages, which points to a link between timing of osteogenesis and ossification (Trueb and Alberch, 1985; Weisbecker and Mitgutsch, 2010).

Bone formation in the anuran larvae has been described to proceed by formation of osteoid matrix first, with subsequent calcification at later stages (Hanken and Hall, 1988), although this has been documented in a single species to date. In addition, not all the skeleton necessarily ossifies at the same moment, with an important decoupling between cranial and post-cranial ossification having been previously documented (e.g., Dunlap and Sanchiz, 1996). Particularly, Dunlap and Sanchiz (1996) found a delayed pattern of cranial ossification relative to the appendicular skeleton in two bufonid species that they related to a rapid larval develop-

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http://dx.doi.org/10.1016/j.jcz.2017.03.002
0044-5231/

ment; a hypothesis that remains to be tested. It should be noted, however, that the larval period lengths of these true toads are not especially short, as shown by the comparative data now available (e.g., Fabrezi, 2011).

The Common Toad *Rhinella arenarum* (Hensel, 1867) (Bufonidae) is a large true toad (88–112 mm; Cei, 1980) from southern South America with a generalized pond-type larva (Altig and Johnston, 1989; Vera Candioti, 2006) and a heavily ossified adult skeleton (Pérez Ben et al., 2014). Together with nearly a hundred species in the same genus, it is part of a widely distributed neotropical radiation that includes most of the former South American *Bufo* Garsault, 1764 (Frost, 2016). This large toad is a common species across most of its wide range and it is a model organism, either at embryonic, larval or adult stages, for ongoing research on histology, endocrinology, ecotoxicology, and cognition, among other topics (e.g., Attademo et al., 2017; Hermida and Farías, 2009; Pollo et al., 2015; Regueira et al., 2013a,b, 2016, 2017; Sotelo et al., 2015). Despite this, there is a remarkable lack of information about its skeletogenesis.

Here we study the sequence and timing of ossification of the entire skeleton of *R. arenarum* through examination of histological sections and cleared-and-stained specimens of an ontogenetic series ranging from early larval stages to juveniles, taking into account intraspecific variation. We aim to explore if osteoid matrix formation and bone matrix calcification are temporally decoupled and to examine the patterns of cranial relative to the appendicular ossification in this species. Finally, we summarize comparative data of a diverse sample of anurans in order to test previous hypotheses regarding the link between delayed cranial ossification and larval period length and to contextualize our observations in a broad phylogenetic context.

2. Materials and methods

2.1. Animals

An ontogenetic series of larvae and juveniles of *R. arenarum* was obtained mostly from wild-caught specimens from temporary ponds at Ciudad Universitaria, Buenos Aires, Argentina. To complete this series, tadpoles were reared at the laboratory in dechlorinated tap water under a natural photoperiod and temperature and fed *ad libitum* with boiled chard. The larvae were staged according to the developmental scheme of Gosner (1960) and the postembryonic larval period was divided into premetamorphosis, prometamorphosis, and metamorphosis (= metamorphic climax) following Etkin (1932). After metamorphosis, newly metamorphosed toadlets were reared under natural outdoor conditions for 6–10 days. Additionally, we studied larvae of *Leptodactylus latinasus* Jiménez de la Espada, 1875, which skeletal ossification have previously been studied (Fabrezi, 2011; Supplementary data), that were collected from the same pond as some of the larvae of *R. arenarum* and reared in the same conditions, in order to lessen potential environmental biases on the timing of ossification. The rationale behind this comparison is that, if a pervasive environmental effect on the ossification is present, whatever it is, it would equally affect both species.

Animals were euthanized by immersion in 0.1% aqueous solution of MS222 (tricaine methanesulfonate; Sigma-Aldrich, St. Louis, MI). The ontogenetic series of *R. arenarum* consists of a total of 130 specimens, including tadpoles of every Gosner stage (hereafter GS) ranging from GS25 to GS45, newly metamorphosed toadlets (GS46), and juveniles (J), whereas the series of *L. latinasus* include 26 specimens representing all stages from GS33 to GS46 (Supplementary data). All the specimens were deposited at the herpetological collection of Laboratorio de Biología de Anfibios, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina. This study was carried out in accordance with the regulations specified by the Institutional Animal Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (Res C/D 140/00) and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The Conservation category of *R. arenarum* and *L. latinasus* is "Least concerned" according to the IUCN Red List criteria (IUCN, 2016) and Vaira et al. (2012).

2.2. Histological and histochemical techniques

Three to seven specimens per stage of *R. arenarum* (99 specimens in total) and one to three specimens per stage of *L. latinasus* (26 specimens in total) were fixed and stored in 10% buffered formaldehyde and cleared and double-stained for bone (Alizarin Red) and cartilage (Alcian Blue) following Wassersug (1976). Specimens were examined under a Zeiss Stemi SV-11 stereomicroscope and photographed using a Nikon D3200 digital camera equipped with a macro lens.

For histological serial sections of *R. arenarum*, 31 animals of selected stages (GS27–46; Supplementary data) were fixed in Bouin's solution for 24 h, embedded in paraffin, and then sectioned in a transverse or sagittal plane at 6 μm (Kiernan, 1999). Sections were stained with modified Masson's trichrome (MMT) stain for general cytology and histology (Regueira et al., 2016). MMT is different from the original Masson's trichrome in that the acid fuchsin solution (0.03% p/v) also contains orange G (0.07% p/v) and xylidine ponceau (0.07% p/v). MMT stained osteoid matrix vividly blue, due to aniline blue affinity for collagen fibers. In addition, von Kossa method was performed to clearly detect calcified matrix (brown; Kiernan, 1999). Histological sections were studied and photographed using a Zeiss Primo Star microscope with an attached Canon PowerShot A640 digital camera.

2.3. Ossification data, criteria and indexes

We accounted for the sequence (i.e., relative order of appearance) of 18 cranial, three axial, and 16 appendicular bones, as well as for their timing of ossification according to Gosner stage, based on examination of cleared-and-stained specimens. We distinguished between osteoid matrix formation and calcification of the bone matrix in the ossification process, but we subsequently referred to as timing of ossification to the onset of calcification to make our observations comparable with most previous works, which reported ossification by recognizing retention of Alizarin Red stain in whole-mounted cleared-and-stained specimens. In addition, we took into account individual variation by means of two different criteria defining the onset of ossification of each bone (Sheil et al., 2014). The onset was determined as the earliest stage at which calcification is apparent: (1) in at least a single specimen (referred hereafter as first-appearance criterion); (2) in 100% of available specimens (referred hereafter as 100% criterion).

The extent of cranial and appendicular ossification during ontogeny was quantified by calculating ossification indexes based on the percentage of elements per skeletal region that were ossified at each particular stage (Supplementary Table S1). For these indexes we computed as one element those bones that are paired (e.g., frontoparietal, femur), serial (e.g., vertebral centra, metatarsals), or form complexes (e.g., radio-ulna, carpals). In view of different approaches observed in the literature to account for individual variation (Dunlap and Sanchiz, 1996; Hanken and Hall, 1984; Sheil et al., 2014), this ossification index was calculated for each stage in three different ways: (1) frequency of element categories (e.g., frontoparietal) that show ossification under the first-appearance criterion (OI1); (2) frequency of element cate-