Sequence of chondrocranial development in the oriental fire bellied toad *Bombina orientalis*

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Abstract
The vertebrate head as a major novelty is directly linked to the evolutionary success of the vertebrates. Sequential information on the embryonic pattern of cartilaginous head development are scarce, but important for the understanding of its evolution. In this study, we use the oriental fire bellied toad, *Bombina orientalis*, a basal anuran to investigate the sequence and timing of larval cartilaginous development of the head skeleton from the appearance of mesenchymal Anlagen in post-neurulation stages until the premetamorphic larvae. We use different methodological approaches like classic histology, clearing and staining, and antibody staining to examine the larval skeletal morphology. Our results show that in contrast to other vertebrates, the ceratohyals are the first centers of chondrification. They are followed by the palatoquadrate and the basihyal. The latter later fuses to the ceratohyal and the branchial basket. Anterior elements like Meckel's cartilage and the rostralia are delayed in development and alter the ancestral anterior posterior pattern observed in other vertebrates. The ceratobranchials I–IV, components of the branchial basket, follow this strict anterior–posterior pattern of chondrification as reported in other amphibians. Chondrification of different skeletal elements follows a distinct pattern and the larval skeleton is nearly fully developed at Gosner Stage 28. We provide baseline data on the pattern and timing of early cartilage development in a basal anuran species, which may serve as guidance for further experimental studies in this species as well as an important basis for the understanding of the evolutionary changes in head development among amphibians and vertebrates.

**KEYWORDS**
Anura, cartilage differentiation, chondrogenesis

1 | INTRODUCTION

The evolution and development of the craniofacial skeleton, an important evolutionary innovation exclusive to vertebrates, has been of great interest to evolutionary biologists for centuries (de Beer, 1937; Goethe, 1820; Huxley, 1857; Oken, 1807). The vertebrate craniofacial skeleton consists of the dermatocranium, the neurocranium, and the viscerocranium, which are subdivided into dermal and endoskeletal components based on their mode of skeletogenesis (Hall, 2015). The dermatocranium is of mixed origin (neural crest and mesodermal) and its elements, which include most of the plate-like bones, ossify without cartilaginous precursors (Franz-Odendaal, Hall, & Witten, 2006).
The viscerocranium is derived from the neural crest and comprises the mandibular, hyoid and gill arches and their respective derivatives which form the lower jaw and its supporting structures (Cerný, Horáček, & Olsson, 2006; Kuratani, Matsuo, & Aizawa, 1997). The neurocranium is of mixed origin and encapsulates the brain and the sensory organs (Coul, Coltey, & le Douarin, 1992). Viscerocranium and neurocranium together form the chondrocranium whose elements undergo endochondral ossification in most species during further development (Morris-Kay, 2001). Chondrocranical development begins, when mesenchymal cells form an aggregation (Hall & Miyake, 1995). These cells then interact with an epithelium, which is always at the site of future skeletogenesis, to initiate a condensation (Hall & Miyake, 2000). The condensed cells start to differentiate into chondroblasts and during further development into chondrocytes. The chondrocytes undergo maturation which results in cartilage formation (Goldring, Tsuchimochi, & Ijiri, 2006). Predominantly, the cartilaginous chondrocranium is the earliest functional skeletal element to form (Rose, 2009). It enables food acquisition and additional capabilities. Therefore, the proper development of the chondrocranium is fundamental to successful further development.

Investigations on chondrocranial development are available for a growing number of vertebrate species including chondrichthyans (Gillis, Dann, & Shubin, 2009; Gillis, Modrell, & Baker, 2012), sturgeons (Gillis et al., 2012; Warth, Hilton, Naumann, Olsson, & Konstantinidis, 2017), teleosts (Langille & Hall, 1987), coelacanths (Dutel et al., 2019), anurans (Lukas & Olsson, 2018a; Parker, 1876, 1879; Stöhr, 1882), testudines (Kuratani, 1999; Tulenko & Shell, 2007), and squamates (Hernández-Jaimes, Jerez, & Ramírez-Pinilla, 2012; Ollonen, da Silva, Mahlow, & Di-Poi, 2018). Investigating the sequential development of cartilaginous elements in a variety of vertebrate species is essential for an understanding of evolutionary changes and for the identification of ontogenetic novelties as well as heterochronic events. The morphology of the chondrocranium of anuran tadpoles has been described in several species (e.g., Candioti, 2007; Candioti, Haas, Altig, & Peixoto, 2017; Haas, Candioti, & Baldo, 2014; Kolenc et al., 2013). Unfortunately, sequential descriptions of cartilage formation from the onset of chondrification until the premetamorphic stage in anurans are scarce (Lukas & Olsson, 2018a; Reiss, 1997) or outdated (Gaupp, 1906; Parker, 1876, 1879; Stephenson, 1951; Stöhr, 1882; van der Westhuizen, 1961). Nevertheless, such investigations are important for questions regarding the origin of tetrapods and the evolution of morphological diversity of anurans. The foundation for this diversity may be laid during the ontogenetic process of cartilage formation.

The morphology of anuran tadpoles has several unique aspects such as the presence of the infrarostral and suprarostral cartilages as anterior parts of the lower and upper jaw, respectively (McDiarmid & Altig, 1999). Tadpoles of the anuran Bombina orientalis (Boulenger, 1890) display a generalized morphology close to the basal state in anurans (Cannatella & De Sá, 1993; McDiarmid & Altig, 1999) and are well suited for studies of the sequence of skeletal development. B. orientalis has been used for different types of investigations, such as, for example, chimeric experiments (Wagner, 1949, 1959), descriptions of different morphological aspects (Haas, 1997; Ridewood, 1897; Sokol, 1975; Wassersug & Hoff, 1982), embryonic staging (Prema, 1981; Sussman & Betz, 1978), ossification sequences (Hanken & Hall, 1984; Maglia & Pügner, 1998), neural crest extirpations (Olsson, Falcik, Lopez, Cobb, & Hanken, 2001; Olsson & Hanken, 1996), knockdown experiments (Lukas & Olsson, 2018b), toxicological surveys (Park, Song, Kim, & Gye, 2017), and peptide research (Xiang et al., 2017).

B. orientalis belongs to the family Bombinatoridae which is closely related to the Alytidae. Both are part of the Discoglossidae, the second most basal branch of the Anuran phylogenetic tree (Feng et al., 2017). The cranial skeleton of the tadpole of B. orientalis has features typical for Discoglossidae such as the presence of two posterior processes at the pars alaris of the suprarostral cartilage and the reduced urobranchial process (Haas, 2003; Sokol, 1981). In B. orientalis, the suprarostral cartilage articulates with the cornua trabeculae via a synchondrosis (Svensson & Haas, 2005). The larval otic process, one of three processes which anchor the palatoquadrate onto the neurocranium, is flat in B. orientalis tadpoles (Sokol, 1981).

With the present work, we fill in a gap which exists in the scientific record between the description of early Gosner (1960) stages from Go1-20 (Prema, 1981; Sussman & Betz, 1978) and late larval development from Go 35 onward (Hanken & Hall, 1984; Maglia & Pügner, 1998) of B. orientalis. Here, we provide a comprehensive description of the cartilaginous development of B. orientalis tadpoles from the emergence of mesenchymal Anlagen until the premetamorphic cartilaginous head skeleton. In addition, the cartilaginous development of B. orientalis is compared to the development of the derived tadpole of Xenopus laevis (Daudin) to identify evolutionary tendencies of head development within anurans and provide insights into the possible ancestral pattern of cartilage formation.

The results of the present study form an important baseline for experimental studies of the development of the larval head skeleton in this species. We are for example using B. orientalis and other species to investigate the origin of the rostralia using loss- and gain-of-function methods. The interpretation of results from experimental manipulations requires a very good knowledge of normal development, as given by the present work.

2 METHODS

2.1 B. orientalis husbandry and staging

Adult males and females of B. orientalis (Boulenger, 1890) were kept in our departmental breeding facility in mixed groups of 20 animals. From November until February, they were kept at 8°C with minimum food supply to simulate natural behavior. After this cool down, they were kept at 24°C and fed ad libitum until the males started calling. Mating and egg deposition took place in shallow water. Eggs were collected manually and cultured in 0.1X modified Barth’s saline (Klein, 2001). Breeding temperature for different clutches ranged from 18 to 23°C. All embryos and larvae were staged according to the
simplified staging table for anuran embryos and larvae (Gosner, 1960) and denominated as "Go stages." Developmental series from defined stages between Go 19 and Go 35 were taken from the clutch. Anesthesia was performed using 1% tricaine methane sulfonate (MS-222) according to the animal welfare protocols at the Friedrich-Schiller-University Jena. Larvae were fixed in 4% phosphate-buffered formalin (PFA) or in Dent's fixative, depending on the specific further investigation. In total, 196 larvae were used in this study (Table 1). The specimens investigated are listed in Table 1. Slides, cleared-and-stained and whole mount antibody stained larvae are kept at the Institut für Zoologie und Evolutionsforschung, Friedrich-Schiller-University, Jena, Germany.

2.2 | Tissue staining

PFA-fixed specimens were used for serial sectioning. They were dehydrated and embedded in paraffin. Serial sectioning was performed using a rotary microtome (Microm, HM 355 S). Serial sections of 7 μm thickness were collected on microscope slides and stained according to Heidenhain's Azan technique (Heidenhain, 1915). Images were taken with an XC10 Olympus camera mounted on an Olympus BX51 microscope operated with dotSlide software. The clearing-and-staining procedure followed the protocol provided by Dingerkus and Uhler (1977) with the exception, that no alizarin red was used due to the absence of bones. Cleared-and-stained specimens were examined with a Zeiss Stemi 11 and images were taken by an attached camera (ColorView) operated by AnalySIS software. Specimens fixed with Dent's fixative were used for whole mount antibody staining. A monoclonal antibody against collagen II (116B3-collagen II, obtained from the Developmental Studies Hybridoma Bank) and Alexa 568 (Thermo Fischer Scientific) as fluorescent secondary antibody were used to specifically stain cartilages. Image stacks (10 μm z-plane, 1 AU) were produced using a confocal laser scanning microscope (LSM 510, Zeiss).

2.3 | 3D reconstruction

Images of serial sections were stacked with Fiji (Schindelin et al., 2012, RRID:SCR_002285). Stacks were aligned using the least squares (rigid) and the elastic non-linear block correspondence mode from the TrakEM2 plugin for Fiji (Cardona et al., 2012). Aligned serial sectioning stacks and CLSM stacks were segmented using the Amira 6.0.1. 3D-analysis software (FEI Visualization Sciences Group, RRID: SCR_007353) for surface rendering. Surfaces were exported to the Wavefront OBJ file format and further processed using Maya 2020 (Autodesk, Inc.). Pictures were rendered using Autodesk Mudbox2017 (Autodesk, Inc.).

3 | RESULTS

At Go20 B. orientalis, tadpoles are laterally flattened and blood starts to circulate in the gills. The cornea and the tail fin become transparent until Go22 and the tail further elongates (Figure 1a). At Go22, the paired cement glands develop on the ventral surface posterior to the mouth, which begins to open. During further development, the mouth moves anteriorly and at Go35 is placed at the anterior ventral surface (Figure 1e). The gut develops a notch on the left side at Go 22 (Figure 1a). Papillae develop from Go24 onward and the operculum overgrows the gills (Figure 1b) at first on the right then on the left side of the embryo until the gills are covered by stage Go25. The gut develops several slings which twist into each other in a specific way. The head flattens from Go25 onward and becomes much wider. Keratodonts begin to develop and form two rows on the upper lip and three rows on the lower lip during further development. Pigmentation increases at Go26 on the dorsal side but ventrally the tadpole remains translucent. Small limb buds are visible at Go26, grow posteriorly and develop small finger buds until Go35.

As described in X. laevis embryos (Lukas & Olsson, 2018a), the external morphology of tadpoles of the same stage sometimes differs greatly in the progress of skeletogenesis. Conclusions about the skeletal state cannot be drawn from the external development and vice versa. As an example, a Go25 tadpole can be less developed in terms of skeletogenesis than a Go23 tadpole. This can be observed between Stages Go21 and Go28 (Figure 2). From Go29 onward, no such extreme condition was observed. Therefore, we decided to introduce 16 substages for the development between Go21 and 28 (Figure 2). The substages are letter and number coded. It starts with Stage A1 (Anlage 1) which can occur in specimens at Go21 and Go22. At this stage the first mesenchymal Anlagen of the ceratohyal, palatoquadrate, cornua trabeculae, and Meckel's cartilage are present. Each stage is characterized by a unique combination of further mesenchymal Anlagen. No chondroblasts or chondrocytes are present between Stages A1 and A4. After Stage A4, the first mesenchymal Anlage differentiates into a precartilaginous Anlage consisting of chondroblasts. The first precartilaginous Anlagen to appear are the Anlagen of the

| Go stage method | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 |
|-----------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| C               | —  | —  | 7  | 7  | 8  | 9  | 9  | 10 | 10 | 10 | 10 | 5  | 5  | 5  | 5  | 5  | 5  |
| F               | 3  | 3  | 3  | 5  | 5  | 5  | 4  | 4  | 4  | —  | —  | —  | —  | —  | —  | —  | —  |
| H               | 3  | 3  | 5  | 7  | 7  | 5  | 5  | 4  | 3  | 3  | —  | —  | —  | —  | —  | —  | —  |

Note: C, clearing-and-staining; F, fluorescent whole mount antibody staining; H, histology.
processus lateral hyalis and the ceratohyal at Stage D1 (Differentiation 1). No chondrocytes are present between Stages D1 and D2 but mesenchymal Anlagen and chondroblasts can be observed. From Stage C1 (Cartilage 1) and onward cartilages are present consisting of chondrocytes and a perichondrium. The following 10 stages are a distinct combination of observable fully chondrified cartilages which clearly define every mentioned stage. After Stage C10, we follow the simplified table of Gosner starting with stage Go29.

3.1 | Infrarostral cartilage

The infrarostral cartilages of *B. orientalis* are paired. Each infrarostral cartilage is connected to Meckel’s cartilage via the intramandibular joint. Mesenchymal Anlagen of the infrarostral cartilages are within the first skeletal structures (Figure 2). Two far separated cylindrical Anlagen develop ventrally from the oral cavity, median to the Anlagen of Meckel’s cartilage at Stage A2 (Figure 3a). During further development, both Anlagen draw near each other. At Stage C3, chondroblasts become visible and the two Anlagen fuse. The single precartilaginous Anlage is v-shaped and extends between the two Meckel’s cartilages. The anterolateral tips broaden and the chondroblasts condense further. They differentiate into chondrocytes by Stage C8. The chondrocytes are nested in lacunae and surrounded by a clearly distinguishable perichondrium from this stage onward. The infrarostral cartilage articulates on both lateral tips with Meckel’s cartilage via the properly formed intramandibular joint (Figure 7b). Initially, the joint is situated at the laterodorsal tip, but it migrates caudally during further development. Both parts of the infrarostral cartilage are connected at the median plane via a synchondrosis. This synchondrosis regresses until Stage C10 when both infrarostral cartilages are separated again (Figure 6).
3.2 | Suprarostral cartilage

The suprarostral cartilage is a plate-like structure of the upper jaw of the tadpole. The median plate is flanked laterally by a posteriorly proceeding projection with two dorsal processes on each side. During Stage A2, the mesenchymal Anlagen of the suprarostral alae, the lateral projections on each side of the suprarostral cartilage, arise (Figure 7b). They are two transversally oriented rods parallel to the Anlagen of Meckel’s cartilage, but more dorsally situated. The mesenchymal Anlagen of the suprarostral corpus, the median plate-like part of the suprarostral cartilage, develop at Stage A3. The Anlage connects the two Anlagen of the alae and surrounds the anterodorsal surface of the oral cavity. At Stage C1, the anterior and posterior dorsal process of the alae develop (Figure 7c). At Stage C2, the mesenchymal Anlage of the suprarostral corpus fuses to the anterior chondroblasts of the condensed Anlage of the Cornua trabeculae. The suprarostral alae condense through the Stages C3–C7 (Figure 3b) and chondrify at Stage C8. At this stage, the suprarostral corpus starts to condense and its cells differentiate into chondroblasts. The corpus is properly chondrified at Stage C10 and articulates with the two cornua trabeculae via a synchondrosis.

3.3 | Meckel’s cartilage

Meckel’s cartilage is the posterior cartilaginous structure in the larval anuran jaw which articulates with the palatoquadrate via the primary jaw joint formed by the retroarticular process of Meckel’s cartilage and the articular process of the palatoquadrate. The mesenchymal Anlagen of Meckel’s cartilage develop at Stage A1 (Figure 2). Two transversally oriented rods with a median anterior and a lateral posterior tip lateroventrally to the oral cavity can be observed. The Anlagen shorten anterior–posteriorly and condense until chondroblasts differentiate at Stage C1 (Figure 3d). The anterior and posterior tips gradually broaden. Later, the first forms the articulation with the infrarostral cartilage and the latter will develop into the retroarticular process. From Stage C4 on, the precartilaginous Anlage begins to revolve caudally (Figure 7b). Chondrocytes differentiate at Stage C6. The cartilage is now horizontally oriented and is placed between the palatoquadrate and the infrarostral cartilage. At the same stage the retroarticular...
process forms (Figure 3d) and establishes the articulation with the palatoquadrate. During further development from Stage C10 on the cartilage straightens and elongates on the median side while partially losing its curviness (Figure 6a–d).

3.4 | Palatoquadrate

The palatoquadrate is part of the jaw apparatus and numerous muscles take their origin from this cartilage. It flanks the neurocranium laterally and consists of three processes which connects it to the neurocranium. The appearance of the mesenchymal Anlage of the palatoquadrate, among three other Anlagen, marks the beginning of skeletogenesis in *B. orientalis*. The cylindrical and horizontally oriented Anlage develops at Stage A1 dorsolaterally from the oral cavity between the Meckel’s cartilage and the ceratohyal Anlage. At Stage A2, the Anlage extends medially and is connected to the anlage of the Cornua trabeculae via the developing commissura quadrotocranialis, one of three processes which anchor the palatoquadrate to the neurocranium (Figure 7c). The Anlage broadens laterally and projects
dorsally to form the mesenchymal Anlage of the processus muscularis at Stage A4. Additionally, the Anlage elongates posteriorly and develops a bar of undifferentiated cells. This is the mesenchymal Anlage of the subocular cartilage. The second neurocranial-anchoring process, the processus ascendens, develops at Stage D1 at the posterior tip of the subocular bar Anlage and extends further posterodorsally. Condensation of Anlagen of the different parts of the palatoquadrate takes place in the anterior–posterior direction. Precartilaginous Anlagen consisting of chondroblasts become visible at Stages D2 (palatoquadrate and commissura quadratocranialis), C2 (processus muscularis, subocular cartilage), and C3 (processus ascendens). On the posteroventral surface of the palatoquadrate two tips form and extend ventrally in the direction of the cartilaginous processus lateralis hyalis at Stage C1 (Figure 7c). This is likely the precartilaginous Anlage of the hyoquadrate process which is meant to articulate with the dorsal surface of the ceratohyal. The anterior part of the palatoquadrate and the processus muscularis chondrify at Stage C3 (Figure 4b). At Stage C4, the posterior part of the palatoquadrate

FIGURE 4  B. orientalis, cartilaginous development of the cornua trabeculae (a), the processus muscularis o of the palatoquadrate (b), the subocular cartilage (c), and the ceratohyal (d). Transverse histological sections of Stage A3 (first column), Stage C1 (second column), Stage C5 (third column), and Stage C7 (fourth column) depict the process of chondrification. Difficult observable structures are highlighted by dotted lines. Scale bars indicate 100 μm
is connected to the neurocranium via the precartilaginous processus ascends which extends further dorsally (Figure 7a). At the same time, the subocular cartilage chondrifies (Figure 4c) and the articular process forms on the anterior surface of the palatoquadrate which enables the formation of a proper articulation with Meckel's cartilage at Stage C6. In the following stages, the commissura quadratocranialis (C6) and processus ascends (C7) develop into proper cartilage. Mesenchymal Anlagen of the larval otic process, the third neurocranium anchoring process of the palatoquadrate, arise at Stage C8 at the posterior tip of the subocular bar. They further proceed posteriorly in the direction of the otic capsule and differentiate into chondroblasts at Stage C9. One stage later, this process is chondrified. During further development, it shifts medially and connects the palatoquadrate and the otic capsule.

3.5 | Neurocranium

In B. orientalis, the neurocranium consists of the cornua trabeculæ which are connected to the suprarostal cartilage, the trabeculæ cranii which flank the developing brain laterally, the parachordals which flank the notochord and the otic capsules which contain the auditory organs. Neurocranial development starts with the appearance of paired mesenchymal Anlagen of the cornua trabeculæ lateroventral to the developing brain and laterodorsal to the oral cavity at Stage A1. Development continues in anterior–posterior direction and the Anlagen of the trabeculæ cranii appear at Stage A3, the Anlagen of the parachordals at Stage D1, and the Anlagen of the otic capsules at Stage C2 (Figure 7a). Trabeculæ cranii appear as a posterior outgrowth of the cornua trabeculæ (Figure 7c). Anlagen of the parachordals develop as two spheres lateroventral to the notochord (Figure 7a), and the Anlagen of the otic capsules appear lateroposterior to the parachordals at stage (Figure 7a).

By Stage A4, the cornua trabeculæ proceed between the Anlage of the suprarostal corpus and the commissura quadratocranialis and differentiate into chondroblasts at Stage D2. Both bars of the cornua trabeculæ bend anterolaterally in the direction of the mesenchymal Anlage of the suprarostal corpus. They are connected to the precartilaginous commissura quadratocranialis at Stage C1 (Figure 7c). The trabeculæ cranii further grow posteriorly and differentiate into a precartilaginous Anlage at Stage C1. At Stage C2, the anterior tip of the cornua trabeculæ and the mesenchymal Anlage of the suprarostal corpus fuse (Figure 7c). At the point of the commissura quadratocranialis, two separated bars are visible on each side flanking the brain. The dorsal ones are the cornua cranialis and the ventral ones are the trabeculæ cranii and both bars proceed further posteriorly (Figure 7c). The parachordals grow in anterolateral direction and differentiate into chondroblasts at Stage C3. After that, the trabeculæ cranii and the taenia tecti marginalis fuse posteriorly with the processus ascends and the precartilaginous Anlage of the parachordals at Stage C4. All together, they surround the median basicranial fenestra ventral to the brain. Trabeculæ cranii and cornua trabeculæ chondrify at Stage C5 (Figure 4a) followed by the parachordals at Stage C7 and the otic capsule at Stage C10. At Stage C9, two large foramina are present between the trabeculæ cranii and the taenia tecti marginalis, the anterior foramen opticum and the posterior foramen oculomotoris (Figure 7c). The foramen prooticum is situated beneath the processus ascends. The parachordals are fused medially and each of them bear a small posterior elongated process. Mesenchymal Anlagen of the tectum synoticum, a dorsal bar which connects both otic capsules are present and chondrify until Go32, the same stage when the basicranial fenestra is replaced by cartilage.

3.6 | Basihyal

The nonneural crest derived basihyal (Olsson & Hanken, 1996) is a cylindrical horizontally oriented and small cartilage dorsal to the M. geniohyoideus and anterior to the median range of the ceratohyal. The mesenchymal Anlage of the basihyal arise late during skeletogenesis (Figure 2). It can be observed between the two anterior tips of the two anterior processi of the ceratohyal at Stage C6 as a small and roundish cluster of undifferentiated cells. These cells differentiate into chondroblasts at Stage C9 (Figure 7a,b) and into chondrocytes at Stage C10, as one of the last cartilages which properly form during development. During chondrification, the cartilage elongates laterally and gets close to the median surface of the anterior processi of the ceratohyal.

3.7 | Ceratohyal

The ceratohyal of B. orientalis is a cartilage with several processus: the processus lateralis hyalis, the processus posterior hyalis, the processus anterior hyalis, and the processus anterolateralis hyalis. The ceratohyal initially consists of two cartilages which fuse medially during development and which are additionally connected to the basibranchial. The two mesenchymal Anlagen of the ceratohyal arise between the oral cavity, which is surrounded by the Anlage laterally, and the Anlage of the M. interhyoideus at Stage A1. The Anlage proceeds posteriorly on the median side at Stage A2 which is likely the processus posterior hyalis. The median anterior surface extends cranially which shapes the processus anterior hyalis. The anterior border of the whole Anlage is curved and proceeds far posteriorly on the lateral side and forms the processus lateralis hyalis (Figure 7a,b). Both Anlagen fuse at Stage A4 and a mesenchymal pars reuniens develops on the median line of the united Anlage. By Stage D1, ceratohyal and its processus lateralis hyalis condense and differentiate into chondroblasts. The processus lateralis hyalis is the first part of the whole chondrocranium which properly chondrifies at Stage C1 followed by the main body of the ceratohyal at Stage C2 (Figure 7b). The processus lateralis further proceeds posteriorly. At the same stage, the processus posterior hyalis, which extends posteriorly in the direction of the developing branchial basket, forms a precartilaginous Anlage and later chondrifies at Stage C4. The Anlage of the processus anterior hyalis condenses at Stage
C1 and later chondrifies, as the last part of the ceratohyal, at Stage C7. The continuous anterior border of each side of the ceratohyal bulges in the middle from Stage C9 onward and develops an anterior oriented spike which is the processus anterolateralis hyalis. The processus lateralis hyalis initially proceeds far anterior overlaying the lateral side of the ceratobranchial I, before it extends more laterally (Figure 6a–d). Toward the end the ceratohyal extends over the whole width of the animal and is horizontally oriented and no longer oblique (Figure 6d).

3.8 | Basibranchial

First mesenchymal Anlagen of the basibranchial arise at Stage A2 between the mesenchymal Anlagen of the ceratohyal in the midline of the embryo (Figure 7a,b). The Anlage elongates caudally. At Stage A4, the same stage when both mesenchymal Anlagen of the ceratohyal fuse, the mesenchymal Anlage of the basibranchial is connected to the ceratohyal Anlage. The basibranchial condenses and consists of chondroblasts at Stage D2. It further elongates caudally until it

![Figure 5](image-url)  
**FIGURE 5**  *B. orientalis*, cartilaginous development of ceratobranchial I–IV of larvae. Transverse histological sections of Stage A3 (first column), Stage C1 (second column), Stage C5 (third column), and Stage C7 (fourth column) show the chondrification process of ceratobranchial I (a), ceratobranchial II (b), ceratobranchial III (c), and ceratobranchial IV (d). Note the strict anterior-to-posterior direction of development. Difficult observable structures are highlighted by dotted lines. Scale bars indicate 100 μm
reaches the newly formed mesenchymal Anlage of the hyobranchial plate at Stage C2 (Figure 7b). The chondroblasts are replaced by chondrocytes at Stage C3, therefore the basibranchial is the third cartilage, after the ceratohyal and the palatoquadrate, to form during ontogeny. The basibranchial fuses to its surrounding mesenchymal cells of the hyobranchial plate at Stage C4, when the hyobranchial plate cells differentiate into chondroblasts. From now on, the basibranchial connects the pars reuniens of the ceratohyal and the hyobranchial plate of the branchial basket during further development. No urobranchial process was observed in the specimens investigated.

3.9 | Branchial basket

The branchial basket of *B. orientalis* is composed of four ceratobranchials on each side, a median hypobranchial plate, which connects both quartets of ceratobranchials, and two hypobranchial I on the cranial surface of the hypobranchial plate. The first mesenchymal Anlagen of ceratobranchials I and II are present at Stage A2 as two thin, bent rods posterior to the mesenchymal Anlage of the processus lateralis hyalis (Figure 5a,b). At Stage A3, the mesenchymal Anlagen of ceratobranchial III (Figure 5c, and at Stage A4, the mesenchymal Anlage of ceratobranchial IV, arise posterior to the existing ceratobranchial Anlagen. At later stages, the Anlagen of all ceratobranchials become stouter and bend laterodorsally (Figure 7b). The size of ceratobranchial I–IV decreases in anterior–posterior direction. At Stage C2, the mesenchymal Anlage of the hypobranchial plate develops and connects both ceratobranchials I and the basibranchial. Anteriorly, two processes proceed between the Basibranchial and the processus posterior hyalis. At Stage C3, the ceratobranchial I and II condense and develop into a precartilaginous Anlage followed by the hypobranchial plate at Stage C4 (Figure 7b). This Anlage is also cranially connected to the cartilaginous basihyal. The mesenchymal Anlagen of ceratobranchials III and IV fuse to the precartilaginous Anlage of the hypobranchial plate and both ceratobranchials are connected to each other by the commissura proximalis III. Ceratobranchial II is the only one that is not fused to the hypobranchial plate at this stage. The terminal commissures between the distal tips of each ceratobranchial develop in anterior to posterior direction from Stage C3 until Stage C4. First, the commissura terminalis I develops as a posterior outgrowth from ceratobranchial I as a small precartilaginous rod, which connects it distally with the ceratobranchial II. The commissura terminalis II between ceratobranchials II and III develops in the same way, but the posterior part, which reaches ceratobranchial III still consists of mesenchymal cells instead of chondroblasts. Commissura terminalis III between ceratobranchial III and IV is the last one which develops as a small and short rod of mesenchymal cells. Ceratobranchials I and II are connected proximally by the commissura proximalis I at Stage C5. At the same stage, ceratobranchials II and III are connected through the commissura proximalis II. At Stage C6, ceratobranchial I chondrifies and the chondrification of the branchial basket continues in anterior to posterior direction (Figure 5a–d). At the same time, the connection between ceratobranchial I and the hypobranchial plate declines and will be totally lost by Stage C7. At this stage, ceratobranchials II and the commissurae terminalis and proximalis I chondrify and ceratobranchial II also loses its connection to the hypobranchial plate by Stage C8. The hypobranchial plate develops a distinctive shape until chondrification at Stage C9 with two anterior bulges which are flanking the basibranchial on each side. This anterior part is meant to be the hypobranchial I. A syndesmotic connection between hypobranchial I and ceratobranchial I was not observed. Ceratobranchial III as well as commissurae terminalis and proximalis II chondrify at Stage C9. The chondrification of ceratobranchial IV and commissurae terminalis and proximalis III takes place at Stage C10. After Stage C10, a small projection, the processus anterior brachialis, at the anterior and median surface of ceratobranchial I develops. The dorsal surface of each ceratobranchial unevenly bulges during further development (Figure 6). In the center of the commissurae terminalis I and II, the dorsally proceeding and

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**FIGURE 6** *B. orientalis*, development of the cartilaginous head skeleton of larvae after all major cartilages are present. Shown are cleared and stained specimen of stage Go28 (a), Go30 (b), Go32 (c), and Go 35 (d). Scale bars indicate 1 mm. ap, ascending process; bb, Basibranchial; cbl, ceratobranchial II; ch, ceratohyal; cm, Meckel’s cartilage; ct, cornua trabeculae; ir, infrarostral cartilage; plh, hypobranchial plate; pmp, processus muscularis palatoquadrate; oc, otic capsule; sc, subocular cartilage; sra, suprarostral alae
proximally curved commissurae craniobrachialis I and II develop gradually from Go33 until Go35 (Figure 6c,d).

4 | DISCUSSION

We have presented a comprehensive overview of the chondrogenesis of all major components of the larval head skeleton of *B. orientalis*, ranging from the appearance of early mesenchymal Anlagen until the fully cartilaginous premetamorphic state. We have shown that Stages Go21–Go28 are key stages for *B. orientalis* cartilaginous development, and that the skeletal developmental state of the embryo does not necessarily correspond to the development of external features, as described before in *X. laevis*. To address this point, we presented 16 substages which subdivide the respective Gosner stages. These substages are each a combination of different developmental states of different cartilages by which means every substage can be clearly defined (Figure 7).

4.1 | Developmental sequence of cartilage formation

As described in *X. laevis*, the development of the viscerocranium of *B. orientalis* also does not follow the strict anterior–posterior direction described in other vertebrate taxa (Gaupp, 1906; Gillis et al., 2012; Langille & Hall, 1987; Stöhr, 1882; Warth et al., 2017). The ceratohyal and its lateral process is the first cartilage which chondrifies during development of *B. orientalis* followed by the anterior part of the palatoquadrate and the basibranchial. The early development of the ceratohyal in both, *X. laevis* and *B. orientalis* (Figure 8), may be linked to its importance as a buccal pump. This mechanism draws water into the buccal cavity by bending the medially fused ceratohyals along the longitudinal axis (McDiarmid & Altig, 1999). The importance of an early establishment of a proper inspiration and expiration mechanism mediated by the ceratohyal, may be the driving factor for the early development of this cartilage. Given the fact that *B. orientalis*, which is among the phylogenetically most basal anurans, together with *X. laevis* share the early development of the ceratohyal this may be an apomorphic trait at least for all anurans except the two most basal groups Ascaphidae and Leiopteridae.

Anterior parts of the viscerocranium, such as the suprarotostal and infrarostral cartilages, which are also anuran-specific novelties, do not follow the anterior to posterior sequence of viscerocraniot development in *B. orientalis*, just like in *X. laevis* (Figure 8). These cartilages arise relatively late after more posterior situated cartilages are long established. This supports the hypothesis that the evolution of these novel cartilages may have evolved through an alteration of the ancestral pattern. Recent findings indicate that a gene called zax is involved in the evolution of the infrarostral cartilage of larval anurans (Lukas, Schmidt, & Olsson, 2019). This gene has a common function with its paralogous gene xbap in maintaining cell-free spaces at the position where joints will develop (Lukas & Olsson, 2018c). The incorporation of this gene into the gene regulatory network may have delayed the development of the infrarostral cartilage. An identical process may be the reason for the relatively late development of the suprarotostal cartilage. Another possibility is because larval anurans can develop for a long time using the energy saved in the ventral yolk mass. If enough energy is provided for the development up to a certain stage, the developmental process of establishing cartilage responsible for feeding may not be crucial until the energy is used up completely. Therefore, more important processes like enabling proper respiration could be preferred during skeletal development. Thus, the constraints of different essential processes may have driven the alteration of the ancestral anterior–posterior pattern, delaying the development of the novel anterior cartilages. In contrast to *X. laevis*, where the infrarostral cartilages arise as a single mesenchymal Anlage and stay single through development, there are two distinct mesenchymal Anlagen of each infrarostral cartilage in *B. orientalis*. They fuse during chondroblast differentiation and become separated after the cartilage is established. A common feature can therefore not be derived, and further investigations are needed to trace back the ancestral pattern of infrarostral cartilage development. In accordance with Haas (Haas, 2003) but unlike Maglia, Pügener, and Trueb (2001), we find no evidence for the presence of an admandibular cartilage anterior to Meckel’s cartilage in the specimens investigated.

The branchial basket of *B. orientalis* develops in anterior–posterior direction as described in other anuran larvae (Lukas & Olsson, 2018a; Stöhr, 1882). In *X. laevis*, the ceratobranchials I-V develop consecutively and are connected first medially by the hypobranchial plate and later by the simultaneously chondrifying commissurae terminalis I–III. In *B. orientalis*, the anterior–posterior pattern of branchial basket development is more rigorous. The commissurae terminalis I–III develop gradually and the hypobranchial plate arises at the same time as the ceratobranchial III. The only thing breaking this strict pattern in *B. orientalis* is the relatively late development of the processus anterior-brachialis. As reported by Haas, no spicula I–III are present in tadpoles of *B. orientalis* (Haas, 2003). At the anterior tip of the hypobranchial plate, a hypobranchial I is clearly distinguishable, but no syndesmotic connection occurs between hypobranchial I and ceratobranchial I other than described before (Haas, 1997). The presence of an urobranchial process has been reported in *B. orientalis* (Maglia & Pügener, 1998), but we cannot confirm this in any of the specimens investigated.

The parachordals are among the earliest cartilages to develop in many vertebrates (Langille & Hall, 1987; Stöhr, 1882; Warth et al., 2017). In *X. laevis* as well as in *B. orientalis*, they are the second cartilaginous structure of the neurocranium to chondrify. In both species, the parachordals proceed anteriorly to reach the anterior–posterior developing trabeculae cranii, which resembles a pattern seen in sturgeons (Warth et al., 2017). The three processes anchoring the palatoquadrate to the neurocranion develop in a strict anterior–posterior sequence: first the commissura quadratocranialis, second the processus ascendens, which is combinatorid-typic with a high insertion, and third the flat larval otic process. This specific order of
the developing processes of the palatoquadrate was already observed in *X. laevis* and in *Ascaphus truei* (Lukas & Olsson, 2018a; Reiss, 1997).

The present investigation confirms various discoglossid traits in *B. orientalis*. Tadpoles possess a suprarostral cartilage with two posterior processes, the suprarostral cartilage articulates with the cornua trabeculae via a synchondrosis which occurs during ontogenesis after both cartilages initially develop separated and a flat larval otic process. Additionally, an urobranchial process and spiculae I–III are absent. After the comparison of the timing of cartilage formation in two...
phylogenetically basal anuran species, we can hypothesize the following features of cartilage formation in anurans.

1. The mesenchymal Anlage of the ceratohyal is the first Anlage to appear during development and the ceratohyal is the first cartilage which chondrifies.

2. Neurocranial structures chondrify in anterior–posterior direction.

3. The neurocranium-anchoring processes of the palatoquadrate chondrify in anterior–posterior direction. First the commissura quadratocranialis, then the processus ascendens and last the larval otic processus.

4. Ceratobranchials develop in an anterior–posterior sequence.

5 | CONCLUSIONS

With developmental morphological studies, we gain insights in the phylogeny, in the developmental timing of different morphological traits, and in the homology of different elements. In this work, we provide a comprehensive overview on the cartilaginous development of larval *B. orientalis*. We identified crucial steps in cartilage formation and hypothesized features of cartilage formation for all anurans. Our data support an early development of the ceratohyal as basal trait of larval anurans. We further show that ceratobranchials I–IV and the neurocranium anchoring processes of the palatoquadrate chondrify in anterior–posterior direction. All results together are an important baseline and anatomical framework for prospective experimental studies of *B. orientalis* development as well as for further skeletal development studies in other anuran species.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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