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Untangling the morphological contradiction: First ontogenetic description of the post-hatching skeleton of the direct-developing frog *Brachycephalus garbeanus* Miranda-Ribeiro, 1920 (Amphibia: Anura: Brachycephalidae) with comments on the genus miniaturization

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ABSTRACT

The study of ontogenetic series facilitates understanding developmental and evolutionary patterns and processes amongst vertebrate lineages, being widely explored in this taxon over the last decades. However, developmental data for osseous elements of anurans are mostly focused on pre-hatching stages of lineages with a larval stage, with very little data on post-hatching phenotypic transformations, especially in direct-developing anurans. Previous osteological studies of the miniaturized genus *Brachycephalus* have considered the taxa as a morphological contradiction, given the existence of both paedomorphic and peramorphic traits on the skull and other post-cranial elements. However, none of them have provided a full post-hatching developmental variation of the skeleton for any species of the genus, and thus the observation of such heterochronic changes has remained underexplored. This study aims to provide a detailed developmental description of the osteology of skull and post-cranium osteology of *Brachycephalus garbeanus* based on an ontogenetic series of 16 post-hatched individuals. Our results show that several skull and post-cranial elements are not fully developed after hatching, with complete bone ossification and fusion occurring at later developmental stages. The combination of our data with previous studies also reinforces the occurrence of delayed skeletal development — i.e., ossification — in *Brachycephalus* when compared with other brachycephaloids. We also discuss the emergence of morphological novelties — as the skull and axial plates — and structural simplification — as digit reduction/loss — as possible consequences of the extreme miniaturization of the genus.

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1. Introduction

Evolutionary biologists have historically focused on studies related to chondrification and ossification sequences of vertebrates, with a special interest in understanding the morphological evolution of these elements (e.g., Bardeen 1905; Khan Noon & Evans 2015; Ortíz-Galindo et al., 2008; Starck 1993; Vera Candioti et al. 2020). Since the mechanisms of morphological development of structures are highly conserved amongst vertebrates (e.g., skull

[Duellman & Trueb 1994; Paluh et al. 2020; Vidal-García and Scott Keogh, 2017]), subtle changes in the structure size, shape, and/or ornamentation may result in different phenotypic patterns (see Dos Reis et al., 2020). Ontogenetic descriptions assessing developmental patterns of species are fundamental in understanding the role of ontogeny on generating evolutionary changes within a lineage, especially when considering such changes under a phylogenetic context (Goldberg et al. 2012; Schoch 2006). The comparison of ontogenetic series of three-dimensional structures — especially bones — between closely related taxa provides valuable data on the influence of phylogenetic history, environmental niche adaptation, and biomechanics of different phenotypes (Campos et al. 2010; Vera Candioti et al. 2020). Furthermore, the concept

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of heterochrony — i.e., change in developmental rate or timing — is fundamental when addressing the discussions on the interaction of evolution and development (Gould 1977). Several parameters might be approached for investigating heterochronic changes, such as size, shape, time, sexual maturity, and developmental characteristics of individuals of a lineage (Fabrezi et al. 2010). Paedomorphosis and peramorphosis are the main two types of heterochrony that are frequently detected in miniaturized lineages, both of them either leading to the emergence of several morphological novelties and/or structural simplifications (Gould 1977; Hanken & Wake 1993). Miniaturization is a common phenomenon of extreme size reduction has independently evolved numerous times amongst vertebrates and has reached a few extremes in anurans (Hanken & Wake 1993).

Amongst anurans, developmental stages are usually assessed and described for plesiomorphic biphasic development lineages (see Gosner 1960). Direct development is considered to be a derived condition in amphibians and has independently evolved at least eight times amongst anurans (Padial et al. 2014). For lineages with direct development, data on the developmental stages are based on egg and post-hatching development, since the free-living larval phase is lacking, and the ontogenetic trajectory from embryo to post-hatching stages is attained by the yolk supply (see Townsend & Stewart 1985). This kind of development is also associated with several transformations in comparison to a biphasic life cycle, including several changes in embryonic anatomy (Vera Candiotti et al. 2020), for instance, accelerated growth rates (Goldberg et al. 2012). Although many lineages are known to have evolved such mode of development, pre-hatching and post-hatching ontogenetic data are still scarce or absent for many taxa known or presumed to develop directly (Vera Candiotti et al. 2020; Schweiger et al. 2017). For instance, recent studies have contradicted earlier simplistic assumptions that direct-developing species are stereotypical, reporting several dissimilarities amongst the morphology of these lineages (Schweiger et al. 2017). These findings indicate that the effects of direct development on embryogenesis are most likely not consistent among different direct-developing lineages, opening new windows on the knowledge of patterns and processes that generate the adult phenotype (Schweiger et al. 2017).

The superfamily Brachycephaloidea comprises more than a thousand species, which some has direct development and other are suspect to have it (Padial et al. 2014; Frost 2021). However, within this clade, pre- and post-hatching skeletal ontogeny is so far known for very few taxa, including a few species of the genera *Eleutherodactylus* (Dumeril and Bibron, 1841), *Oreobates* (Jiménez de la Espada, 1872), *Haddadus* (Dumeril and Bibron, 1841), *Ischnocnema* (Reinhardt and Lutken, 1862), and *Brachycephalus* (Fitzinger, 1826) (see for example Hanken, 1992; Campos et al. 2010; Meza-Joya et al. 2013; Vera Candiotti et al. 2020). The miniaturized Neotropical genus *Brachycephalus* is currently recognized based on both morphological and molecular data (Condez et al. 2020; Ribeiro et al. 2015), comprising 38 recognized species, all endemic to the Atlantic Rainforest of southern and southeastern Brazil (Frost 2021). *Brachycephalus* species are extremely miniaturized, with adults exhibiting increased cranial ossification as well as retaining a variety of juvenile features (Alves et al. 2006; Bornschein et al. 2016; Condez et al. 2020; Folly et al. 2020; Trueb & Alberch 1985). The presence of new bones (paravertebral and parotic plates — see Campos et al. 2010) on the body dorsum represents one of the features that aids in the distinction of two distinct lineages within *Brachycephalus* (Condez et al. 2020). Within the genus, post-hatching bone development is described exclusively for *Brachycephalus ephippium* (Spix, 1824) (Campos et al. 2010), for which the authors have focused solely on elucidating

the nature of the bony shields associated with the skull and axial skeleton; while pre-hatching data is known exclusively based on a reduced sample of the same species (Vera Candiotti et al. 2020).

Given the importance of understanding the ontogenetic transformations of the skeletal elements of direct-development anurans, this study aims to provide data on the osteology and ontogenetic development of the skeleton of *Brachycephalus garbeanus* (Miranda-Ribeiro, 1920), also representing the first detailed post-hatching developmental description for the genus based on skull, vertebral column and appendicular skeleton. *B. garbeanus* is known exclusively from the municipality of Nova Friburgo, State of Rio de Janeiro (Frost 2021; Pombal 2010) and, in such a considerably limited distribution, the species is considered to be rare, usually found in high-elevation regions, and inhabiting the forest floor (Dorigo et al. 2012; Folly et al. 2021a, 2021b). Given our sample number, our results will also allow us to discuss the developmental changes and their possible relation to sexual dimorphism, as well as possible heterochronic changes associated with the emergence of morphological novelties and simplifications in adults (see Vera Candiotti et al. 2020; Trueb & Alberch 1985).

2. Material and methods

A total of 16 individuals of *B. garbeanus* were collected from the species type-locality in the municipality of Nova Friburgo, State of Rio de Janeiro, Brazil. The individuals were anesthetized and killed with lidocaine 2%, fixed in formaldehyde 10%, and subsequently preserved in 70% ethanol [usual techniques described by McDiarmid (1994)]. The specimens were deposited in the Amphibian Collection of Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil (MNRJ). We have also examined one specimen from the collection of Célio F. B. Haddad, Universidade Estadual Paulista, Rio Claro, Brazil (CFBH). Examined specimens are listed in Appendix 1.

Specimens were sexed according to Folly et al. (2021a), with males being distinguished by the presence of vocal slits and/or the presence of testicles. Aiming to provide an ontogenetic description for which the transformations in bone development are evident, for description purposes we have followed Campos et al. (2010) to classify individuals in categories by using their snout–vent size (SVL) to determine such categories. Categories selected were (i) young, (ii) intermediate and (iii) adult, and are detailed in Table 1. The SVL of each specimen was measured to the nearest 0.1 mm with a digital caliper. Osteological observations and descriptions are based on two types of preparations: (1) cleared-and-stained specimens and (2) high-resolution computed tomography (Micro-CT). Eviscerating, clearing, and bone staining followed Taylor and VanDyke (1985) with modifications. Two adult specimens (MNRJ 51472, 25398) were tomographed with the aid of high-resolution micro-computed tomography (μ CT) scanning procedures using a Bruker Skyscan 1273 at Instituto Alberto Luiz Coimbra de Pós-graduação e Pesquisa de Engenharia (COPPE), Laboratório de Instrumentação Nuclear, Universidade Federal do Rio de Janeiro, Rio de Janeiro. We used high-resolution micro-computed tomography (μ CT) for skeletal visualization but not for cartilaginous or less ossified elements. The scans were conducted using an X-ray beam with 35–40 kV source voltage and 190–200 μ A current. Rotation steps of 0.3–0.4° were used with a frame averaging of 5, recorded over a 180° rotation, resulting in 600–698 projections of 244–1100 ms exposure time each and a total scan duration of 0h:32m:35s–01h:08:01. Micro CT specimens of *B. garbeanus* are available online at morphosource website: MNRJ 51472 (ID: 000359798) and 25398 (000359857). We used CTVox for Windows 64bit version 2.6 (Bruker, μ CT) for 3D visualization and description, and plates were made using Inkscape 1.0.

Table 1

Data on examined specimens of *Brachycephalus garbeanus*. Developmental stages were defined for description purposes and based on individual SVL and gonad examination referring Campos et al. (2010).

Developmental category	Sex	Maturity	SVL (mm)	Vouchers (MNRJ)
Young	Male	Juvenile	8.9, 9.4, 9.8	39611, 92792, 92796
	Female		10.1, 10.4	39614, 93599
Intermediate	Male	Juvenile	10.9, 11.1	92802, 92805
	Female		12.2, 15.1, 15.4	39612, 93598, 93600
Adult	Male	Adult	14.2, 14.6, 14.8	25398, 51472, 92803
	Female		17.1, 18.1	93601-91603

Photos of cleared-and-stained specimens of *B. garbeanus* were acquired with a Leica M205C stereoscope coupled to a DFC 450 camera available at Setor de Herpetologia, Departamento de Vertebrados, Museu Nacional, Universidade Federal do Rio de Janeiro. Terminology of cranial osteology follows Campos et al. (2010); Folly et al. (2021b); Púgener & Maglia (1997); and Trueb (2015); terminology of hyolaryngeal skeleton follows Trewavas (1933), vertebral column follows Campos et al. (2010), pectoral girdle follows Trueb (1973), *manus* and *pes* follow Fabrezi (1992, 1993, 2001).

3. Results

We provide a detailed osteological description of the individuals considering the transformations found in the three categories defined in this study – i.e., young, intermediate, adult (see Table 1). In a few instances, when variation is absent for a specific element from one stage to other, a description will be provided for all stages. A post-hatching skeletal development summary is presented in Table 2.

3.1. Skull morphology and development of *Brachycephalus garbeanus*

Skull plate (Figs. 1–3). In young specimens, the parotic plate is reduced, lacks ornamentation, and does not dorsally cover the squamosal (Fig. 1A and B). The dorsal plane expands medially towards the frontoparietal from young to adults. This plane is twice longer than wide with a concave anterior projection; and thin medial and lateral projections, varying bilaterally in shape in young males. In young females, the medial projection is robust without any concavity. Ornamentation is first seen in intermediate specimens and seems to develop directly from the surface of the plate as spine-shaped processes that cover the entire surface of the plate in later stages (i.e., intermediate and adult specimens). The post-orbital crest is posteriorly projected and is poorly developed, not reaching the occipital condyle.

Dermal investing bones. Nasals (Fig. 1) – In young specimens, these paired bones are rectangular, located at the anterior portion of the skull, lack contact with any other skull element, and are widely separated from each other by a large medial gap (Fig. 1A). The nasals grow anteromedially in intermediate specimens, and in females, a posteromedial projection contacts the frontoparietal in the anterolateral portion of the latter (Fig. 1D). All adult females have very ornamented nasals, which are fused to frontoparietals (Fig. 1F), while adult males might or not (MNRJ 25398) have conspicuous ornamentations. **Frontoparietals (Fig. 1)** – Young specimens have rectangular frontoparietals (about three times longer than wide) with lateral expansions that are wider than the anterior portion of the bone. In these individuals, the frontoparietals lack ornamentation, and are medially separated by a distinct frontoparietal fontanelle (Fig. 1A and B). Intermediate specimens have a thin flange of frontoparietals projected throughout the posterior portion of the orbit which almost contacts the parotic plate, not yet

fused with frontoparietals (Fig. 1C and D). Some intermediate females (MNRJ 39612 and 93598) exhibit sparse spine-shaped ornamentations in the dorsal lamina of these bones. At this stage, the frontoparietal fontanelle is reduced, but still evident (Fig. 1C and D). In adults these bones are flat, lack cranial crests, and are synostosed medially throughout their entire length, completely obscuring the frontoparietal fontanelle (Fig. 1E and F). The *lamina perpendicularis* is well-developed, ventrally trespassing the level of the orbitonasal foramen. The flange projected throughout the orbit is well developed, wide, plate-like, and contacts the parotic plate. Thus, evident dermal bones with dermal ornamentations are only found in specimens herein assigned as adults (Fig. 1E and F; 3E, F). The nasals, sphenethmoid, frontoparietals, squamosals, and the parotic plate are synostosed and ornamented forming a dorsal skull plate only in these categories/individuals. A marginal extension of the frontoparietals is fused to the parotic plate, dorsally covering the exoccipitals and prootics.

Neurocranium. Sphenethmoid (Fig. 2)– In young/intermediate males, and young female specimens this bone is cartilaginous, with two centers of endochondral ossification. The anterior margins of both ossification centers are convex and broader than the posterior (Fig. 2A–C). Dorsally at its anterolateral portion, the sphenethmoid is slightly covered by the nasal, and half of its posterior length is covered by the frontoparietal. Ventrally, the cultriform process of the parasphenoid overlaps around half of its length. In both stages (young and intermediate) the optic foramen is surrounded by cartilage (Fig. 2B). The distal portion of the sphenethmoid is ossified in two intermediate females (MNRJ 93598, 93600). In adult specimens, this bone is almost fully covered by the frontoparietals and nasals in dorsal view. Ventrally, its posteromedial portions are covered by the cultriform process of the parasphenoid and its anterolateral part is covered by the vomers. In adults, the optic foramen is surrounded by bone (Fig. 2E and F).

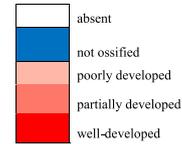
Exoccipitals and Prootics (Fig. 1)– Young specimens have ossified exoccipitals, opisthotic and cartilaginous otic capsule (Fig. 1A and B). Intermediate specimens have ossified oto-occipitals, prootics, well-developed epiotic eminence and otic crests (anterior and posterior; Fig. 1C and D). Two intermediate females (MNRJ 93598, 93600) have such eminences dorsally covered by the skull plate. In adults, the frontoparietal and the parotic plate are fused and it covers the prootic (Fig. 1E and F).

Ventral investing bones. Parasphenoid (Fig. 2)– Young to adult specimens have a T-shaped and fully ossified parasphenoid, with all its processes (i.e. alae, cultriform, and posteromedial) fully developed. The cultriform process invests the sphenethmoid and extends until the anterior first third of the orbit, not articulating with the vomers. This process is anteriorly truncated, being around the width of the occipital condyle throughout its entire length. The parasphenoid alae are thin and perpendicular to the cultriform process, located beneath the otic capsule; the distal margins of the alae are truncated and terminate after the midpoint of the otic capsule. Each ala has around more than 2/3 of the cultriform process length and does not articulate with the medial ramus of the

Table 2

Developmental events during post-hatching development in *Brachycephalus garbeanus*. A reference for colors is given; dark colors show later development of structures, whereas lighter colors are indicative of early differentiation. The categories of development are related to the adult stage phenotype and also level of ossification, with “well-developed” meaning that the bone reached its final morphology.

Skeletal element	Skeletal subelement	Post hatching stages					
		Young		Intermediate		Adult	
		Male	Female	Male	Female	Male	Female
Nasal	-						
	maxillary process						
Frontoparietal	ornamentation	Absent	Absent	Absent	Absent		
	-	Absent	Absent	Absent			
	Frontoparietal fontanelle						
	flange	Absent	Absent				
Sphenethmoid	ornamentation	Absent	Absent				
	-				*slightly ossified		
Oto-occipital	optic fenestra						
Exocoipitals	-						
	epiotic and otic eminence				*might be covered by the skull plate	*covered by skull plate	
Parotic plate	-						
	ornamentation						
Parasphenoid	cultriform process						
	alae						
Neopalatine	posteromedial						
	-	Absent	Absent	Absent	Absent	Absent	Absent
Vomer	anterior process						
	prechoanal process						
	postchoanal process						
	dentigerous process	Absent	Absent	Absent	Absent	Absent	Absent
Premaxillae	-						
	<i>pars palatina</i>						
	alary process						
Maxillae	<i>pars dentalis</i>						
	-						
Quadratojugal	<i>pars facialis</i>						
	-	Absent	Absent	Absent	Absent	Absent	Absent
Pterygoid	-						
	anterior ramus						
	medial ramus						
Squamosal	posterior ramus						
	-						
	ventral ramus						
	zygomatic ramus						
Mandible	otic ramus						
	mentomeckelian						
Columellae	dentary						
	<i>pars articularis</i>	Unknown					
Operculum	-	Absent	Absent	Absent	Absent	Absent	Absent
	hyoid plate						
Hyolaryngeal skeleton	posteromedial processes						
	arytaenoids						
	oesophageal process						
Pectoral girdle	clavicle						
	procoracoid						
	epicoracoid						
Column	Spinal plate	Absent	Absent				
	Spinal plate - ornamentation	Absent	Absent				
	dorsal plate						
	Dorsal plate - ornamentation						
Manus	Sacral diapophyses sesamoids	Absent	Absent	Absent			
	fused distal carpal 5–4–3						
	fused distal carpal 2 and element Y						
	radiale						
	ulnare						
Pes	prepollex						
	tibiale						
	fibulare						
	fused distal tarsal 2–3						
Prehallux	distal tarsal 1						
	element-y						
	prehallux						



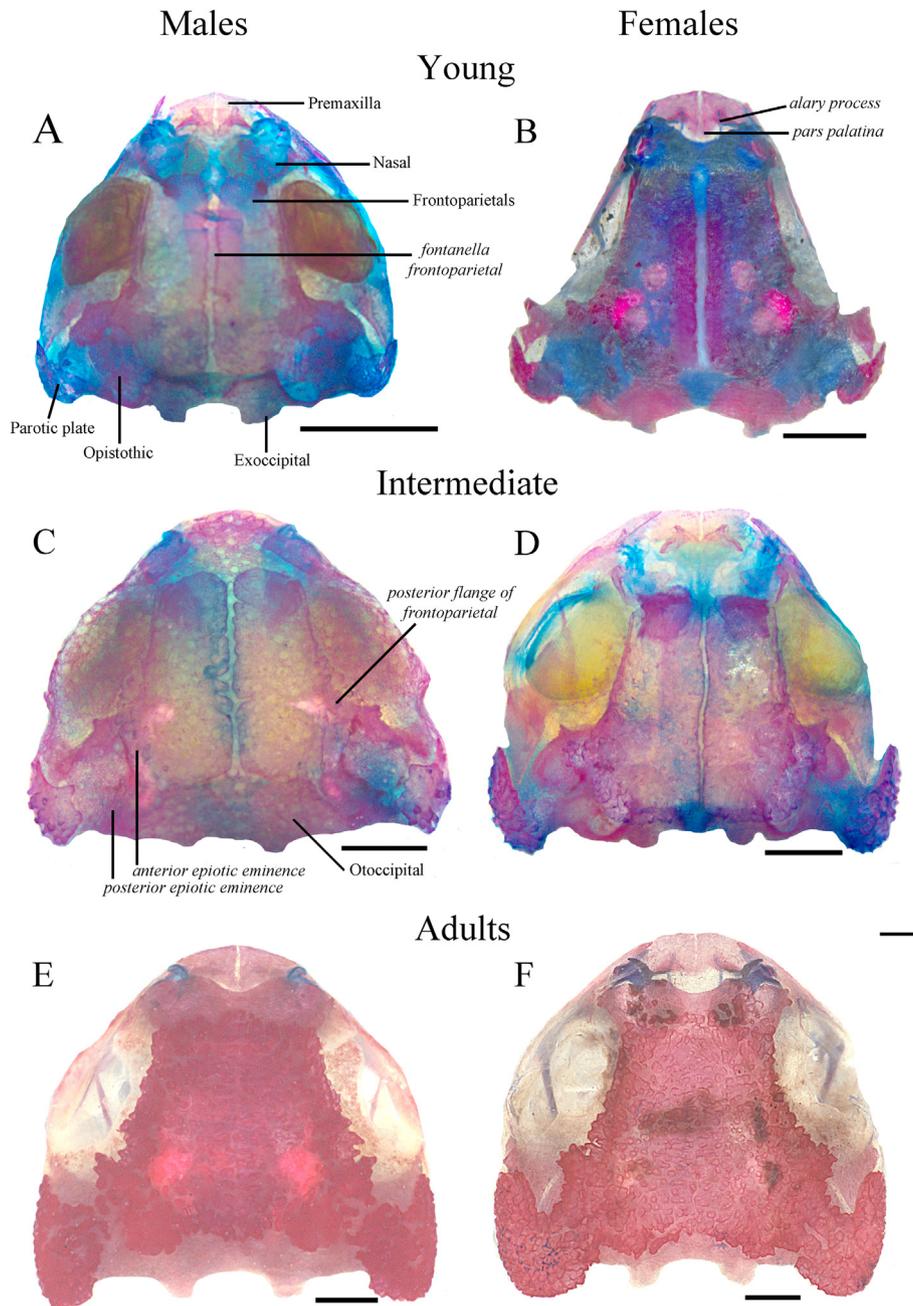


Fig. 1. Ontogenetic post-hatching development of skull (dorsal view) of *Brachycephalus garbeanus*. (A) young male (MNRJ 39611; SVL 9.8 mm); (B) young female (MNRJ 93599; SVL 10.1 mm); (C) intermediate male (MNRJ 92805; SVL 10.9mm); (D) intermediate female (MNRJ 39612; SVL 12.2mm); (E) adult male (MNRJ 92803; SVL 14.8 mm); and, (F) adult female (MNRJ 93602; SVL 18.3 mm). Roman typeface is used to label the bones, whereas italics are used to designate a part of a bone (e.g., ramus, process). Scale bar = 1 mm.

pterygoid. The parasphenoid terminates in a broad triangular posteromedial process that participates in the formation of the foramen magnum. The only observed modification throughout the stages regards the degree of development of the posteromedial process, with young specimens having a slightly triangular process that becomes rectangular in intermediate specimens, later broadening in adult specimens. *Neopalatine* – Absent in all stages. *Vomers* (Fig. 2) – Young and intermediate specimens have reduced semi-lunar vomers that are not fully formed (Fig. 2A and B), and the processes of the vomers in young specimens are indistinguishable. Larger young males have slightly distinguished cartilaginous processes (except dentigerous), which terminate in a rounded tip. Intermediate specimens have more developed processes, although these are still reduced. Adult specimens have anterior processes

with a triangular rounded tip that is as long as the prechoanal process. The posteromedial margins of the vomers are moderately separated from each other, abruptly diverging anteriorly. The pre- and postchoanal processes are around the same length, and the dentigerous process is absent. In adults, the vomers totally fuse to the sphenethmoid (Fig. 2E and F).

Maxillary arcade. Premaxillae (Figs. 1–3) – The general shape of each premaxilla in all stages and specimens is similar, with both alary and palatine processes being present. Each premaxilla is broad and has a rectangular shape with the medial side longer than the lateral, the latter representing the palatine process. The *pars dentalis* lacks teeth, and the alary processes are curved with their basal parts converging medially. In anterior view, the height of the alary process corresponds to around the length of premaxillae, with

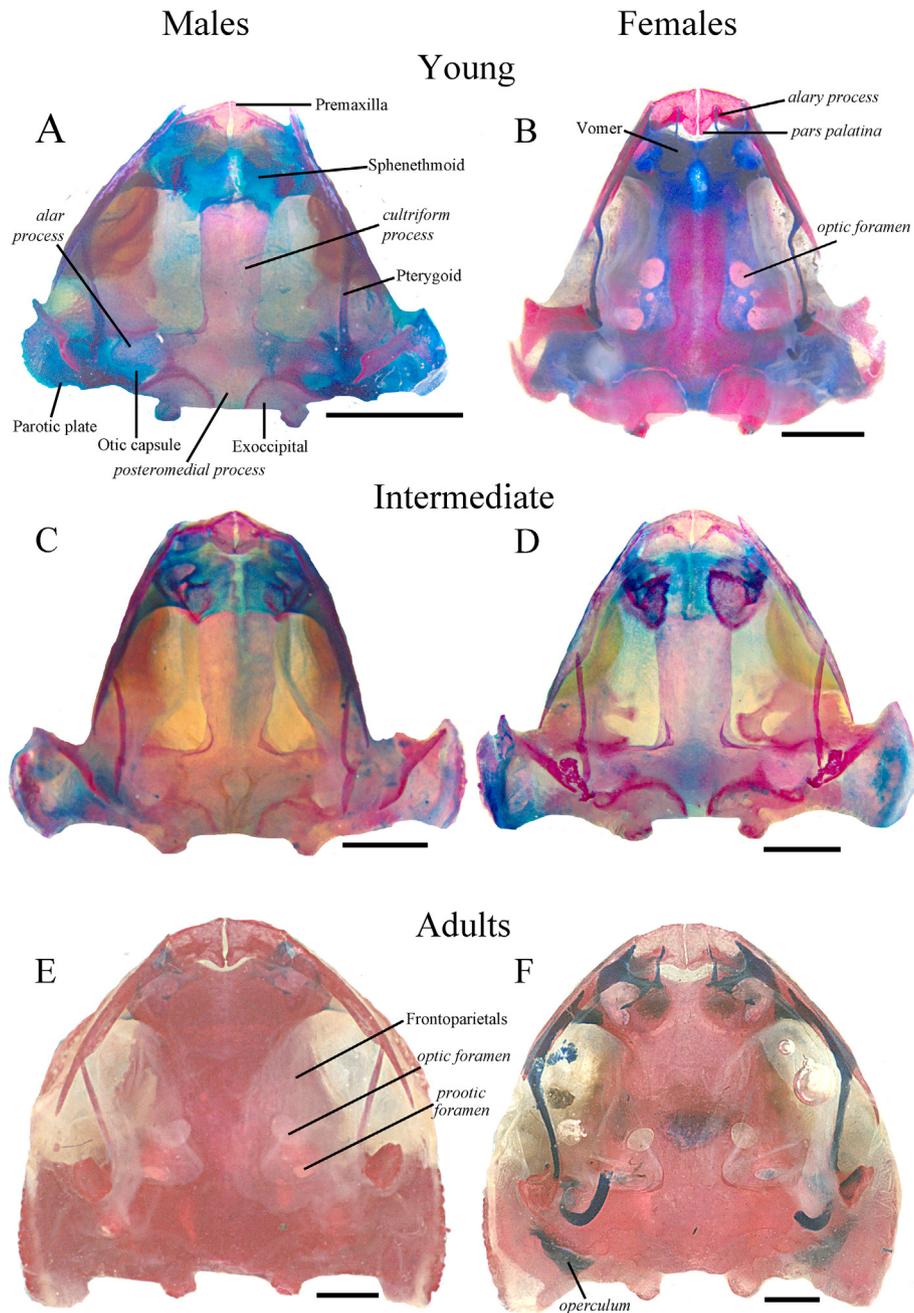


Fig. 2. Ontogenetic post-hatching development of skull (ventral view) of *Brachycephalus garbeanus*. (A) young male (MNRJ 39611; SVL 9.8 mm); (B) young female (MNRJ 93599; SVL 10.1 mm); (C) intermediate male (MNRJ 92805; SVL 10.9 mm); (D) intermediate female (MNRJ 39612; SVL 12.2 mm); (E) adult male (MNRJ 92803; SVL 14.8 mm); and, (F) adult female (MNRJ 93602; SVL 18.3 mm). Roman typeface is used to label the bones, whereas italics are used to designate a part of a bone (e.g., ramus, process). Scale bar = 1 mm.

the distal tip of each alary process being pointed and diverging from one another. The distal end of each *pars palatina* is sharp and converges to each other almost touching one another. *Maxillae* (Fig. 3)– In the smallest young female specimen (see Table 1), this bone has a narrow-angle relative to the frontoparietal, almost parallel with each other and the posterior end is oriented to the oto-occipital lateral margin. Young specimens are toothless, and the posterior end of each maxilla is sharp, in contact with the premaxilla, and does not reach the ventral ramus of the squamosal. The *pars facialis* is high, thin and waved at the anterior side. It extends for less than half the length of the maxilla and is restricted to the level of the maxillary process of nasal, without contacting each other. Adults have a *pars facialis* that contacts the maxillary process

of nasal and the anterior end of the maxilla; this process is overlapped by the posterolateral end of the premaxilla.

Suspensory apparatus. Quadratojugals – Absent in all specimens examined. The *pars articularis* is cartilaginous in young females, being ossified in intermediary and adult specimens. *Pterygoids* (Fig. 3)– These paired bones bear well-developed anterior and posterior processes, and a short medial process. Its morphology in young, intermediate, and adults are alike, being long, cylindrical, terminating in a truncate apex, and extending until the final third of the orbit. The medial ramus is slightly laminar with a truncated end that invests the prootic. The posterior ramus is long, triangular, and has a truncated edge. *Squamosals* (Fig. 3)– This bone is composed of three discernible rami - the ventral ramus, the otic ramus (posterior

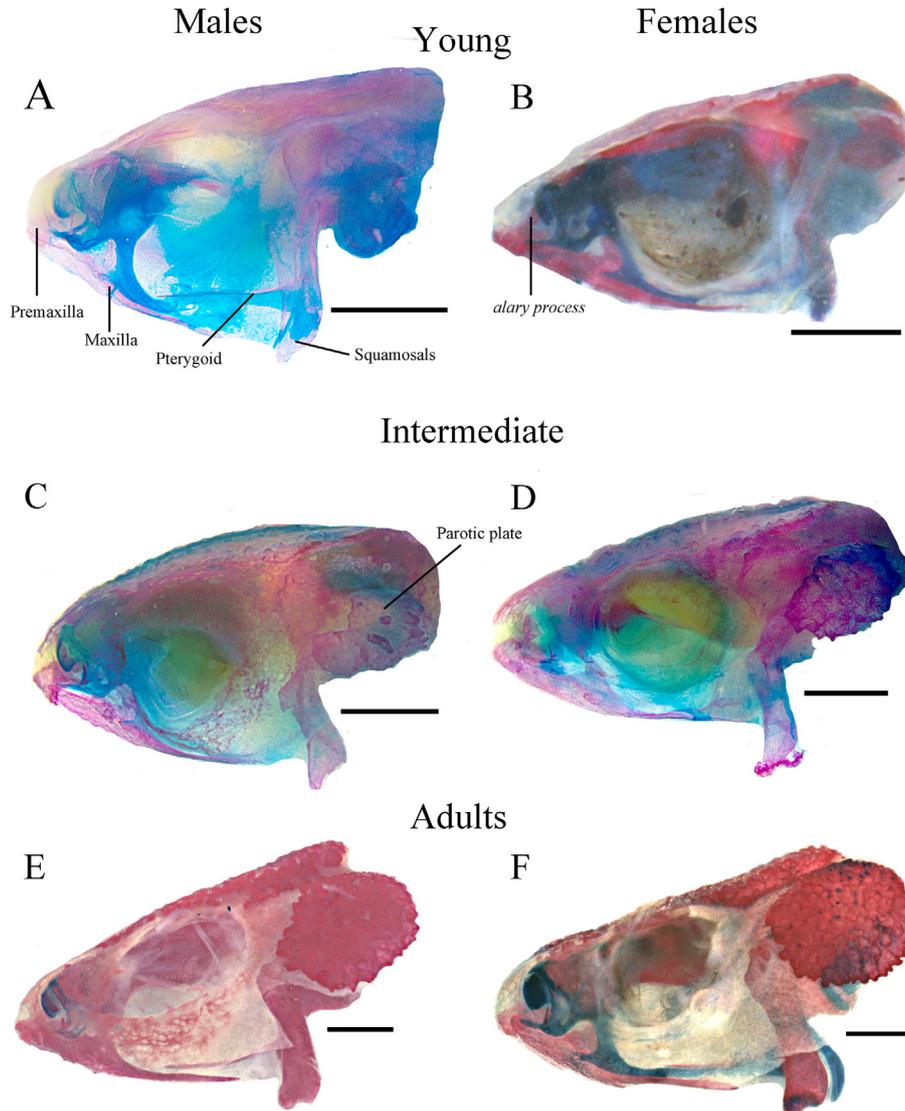


Fig. 3. Ontogenetic post-hatching development of skull (lateral view) of *Brachycephalus garbeanus*. (A) young male (MNRJ 39611; SVL 9.8 mm); (B) young female (MNRJ 93599; SVL 10.1 mm); (C) intermediate male (MNRJ 92805; SVL 10.9 mm); (D) intermediate female (MNRJ 39612; SVL 12.2 mm); (E) adult male (MNRJ 92803; SVL 14.8 mm); and, (F) adult female (MNRJ 93602; SVL 18.3 mm). Roman typeface is used to label the bones, whereas italics are used to designate a part of a bone (e.g., ramus, process). Scale bar = 1 mm.

ramus), and the zygomatic ramus (anterior ramus) - that render the squamosal as a “T”-shaped element. The relative lengths of its rami are ventral > otic > zygomatic. The ventral ramus has a rounded distal end, which is wider than its proximal portion. In lateral view, the zygomatic ramus is reduced, being rounded towards the maxilla. In lateral view, the otic ramus has a rounded distal end. In dorsal view, the distal end of the otic ramus is truncated. Throughout the three developmental stages, the otic ramus is visible in young specimens but partially overlapped by the parotic plate in adults.

Mandible (Fig. 4)— Young males were lacking the mandible due to material preservation issues; thus, we could not describe this feature for these individuals. All stages have mentomeckelian bones in the anterior part of the mandible and are separated from each other by a short space. In anterior view, the mentomeckelians are sharp anteriorly and are laterally fused to the dentaries. Each dentary extends for less than half of the angulosplenic bone and has a pointed posterior end. The anterior end of each angulosplenic is rounded at its anterior tip, and the posterior end is robust and rounded. In one adult CT-scanned specimen, a reduced osseous

element (possibly a sesamoid) is present on each side of the articulation with the angulosplenic. This element is not present in any other specimen and its nature is unclear.

Auditory Apparatus—The columella is absent, and the *fenestra ovalis* is posteriorly oriented. Young specimens and intermediate males have a triangular shaped and cartilaginous *operculum*, which is usually ossified in intermediate and adult females (MNRJ 39612, 93598, 93601, 93603). Adult specimens have enlarged, mushroom-like, and complete ossified *operculum* (except for the female MNRJ 93602) that nearly fills the *fenestra ovalis*.

3.2. Hyolaryngeal skeleton (Fig. 4)

We were unable to observe the hyolaryngeal skeleton for some individuals since this structure for adult and young males were removed/damaged during clearing and staining procedures. In all female stages this structure is similar in shape and exhibits the same degree of ossification, thus a general description is provided herein. The hyoid plates are cartilaginous, longer than wide, with their length around four times wider than its smallest width. The

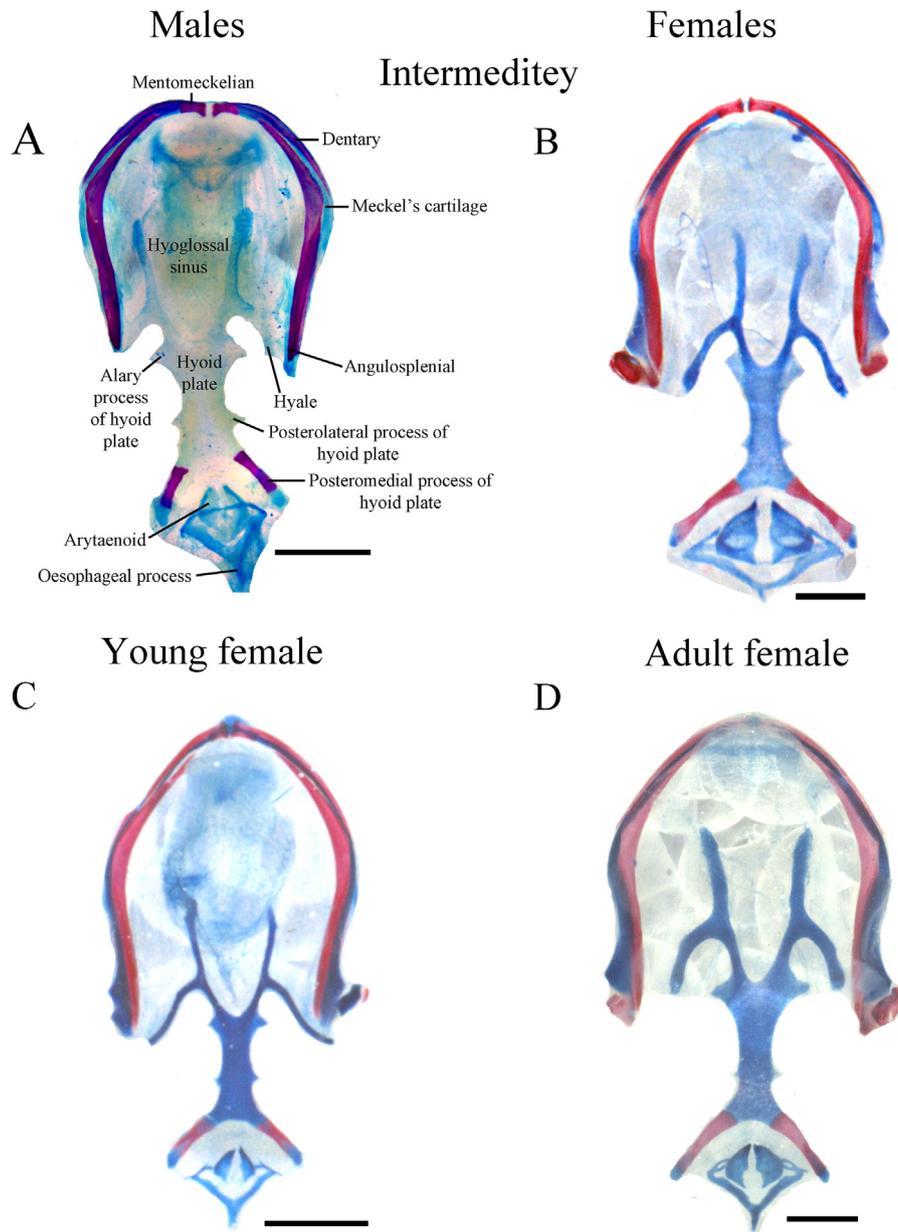


Fig. 4. Ontogenetic post-hatching development of mandible and hyolaryngeal apparatus of *Brachycephalus garbeanus*. (A) intermediate male (MNRJ 92805; SVL 10.9 mm); (B) intermediate female (MNRJ 93600; SVL 15.4 mm); (C) young female (MNRJ 93599; SVL 10.1 mm); and, (D) adult female (MNRJ 93602; SVL 18.3 mm). Scale bar = 1 mm.

anterior processes are long and form a deep hyoglossal sinus, which deepens to nearly the height of the alary processes. The hyoglossal sinus is narrow, U-shaped with approximately the same length as the hyoid plate. Alary processes are around more than half of the hyoid plate. The alary and posterolateral processes are much reduced, with the former being longer than the latter. The ossified posteromedial processes are posterolaterally oriented, widely diverging distally to embrace the broad larynx. Their length is about half of the hyoid plate, being almost totally ossified, except for its distal tip that remains cartilaginous. The arytaenoids are short, semicircular and their posterodorsal ends are widely separated. An apical cartilage seems to be present. The oesophageal process has the same length as the arytaenoids. Laterally contiguous with the posteromedial process, the lateral process of the croid fits over the end of the posteromedial process.

3.3. Postcranium

3.3.1. Vertebral column (Fig. 6)

Vertebrae—All stages have nearly the same general vertebral shape. Observed changes regard the degree of development and fusion of presacral vertebrae to the plates associated with the vertebral column (but see below), and the presence of sesamoids at the sacral diapophyses.

All specimens have a vertebral column composed of eight presacral, non-imbricate vertebrae, sacrum, and urostyle. The presacral-I (=atlas) bears widely spaced cervical cotyles; presacrals II – IV bear moderately stout transverse processes, and presacrals V – VIII bear shorter, less robust processes. Transverse processes of presacrals III – V are curved posteriorly, and transverse processes of the presacrals II, VI – VIII are oriented anteriorly. The relative

lengths of the transverse processes of the presacrals formula follow: IV > III > V > Sacral diapophyses (SD) > II > VI – VIII. Small, slightly ornamented ridges (=paravertebral plate) are noticed at the distal part of the transverse process of vertebra IV in young specimens (Fig. 6A and B). In this stage, the sacral diapophysis sesamoids are absent. Early stages of young males and females bear dorsally unfused neural arches of vertebrae I-III, also being ventrally unfused to the centrum (Fig. 6A and B). In intermediate males, the plate growth is noticed dorsally covering the transverse processes of vertebrae IV-VI, while in females it covers vertebrae II-VI (Fig. 6C and D). Spinal plates are present and not ornamented in

intermediate specimens (Fig. 6C). Intermediate males do not have sacral diapophysis sesamoids, while females have small ones. The sacral diapophyses are moderately expanded and oriented posteriorly, and the distal end of diapophyses remains as a slightly calcified cartilage that articulates with the ilial shaft of the pelvic girdle. Both adult male and female have a dorsal transverse crest at the sacrum, and sesamoids are present on both sides of sacral diapophyses. There are two types of bony elements associated with the vertebral column: (1) spinal plates - dorsal to the vertebra and ornamented, being convex at their dorsomedial portion; (2) the paravertebral plates - a shield that covers a large portion of the

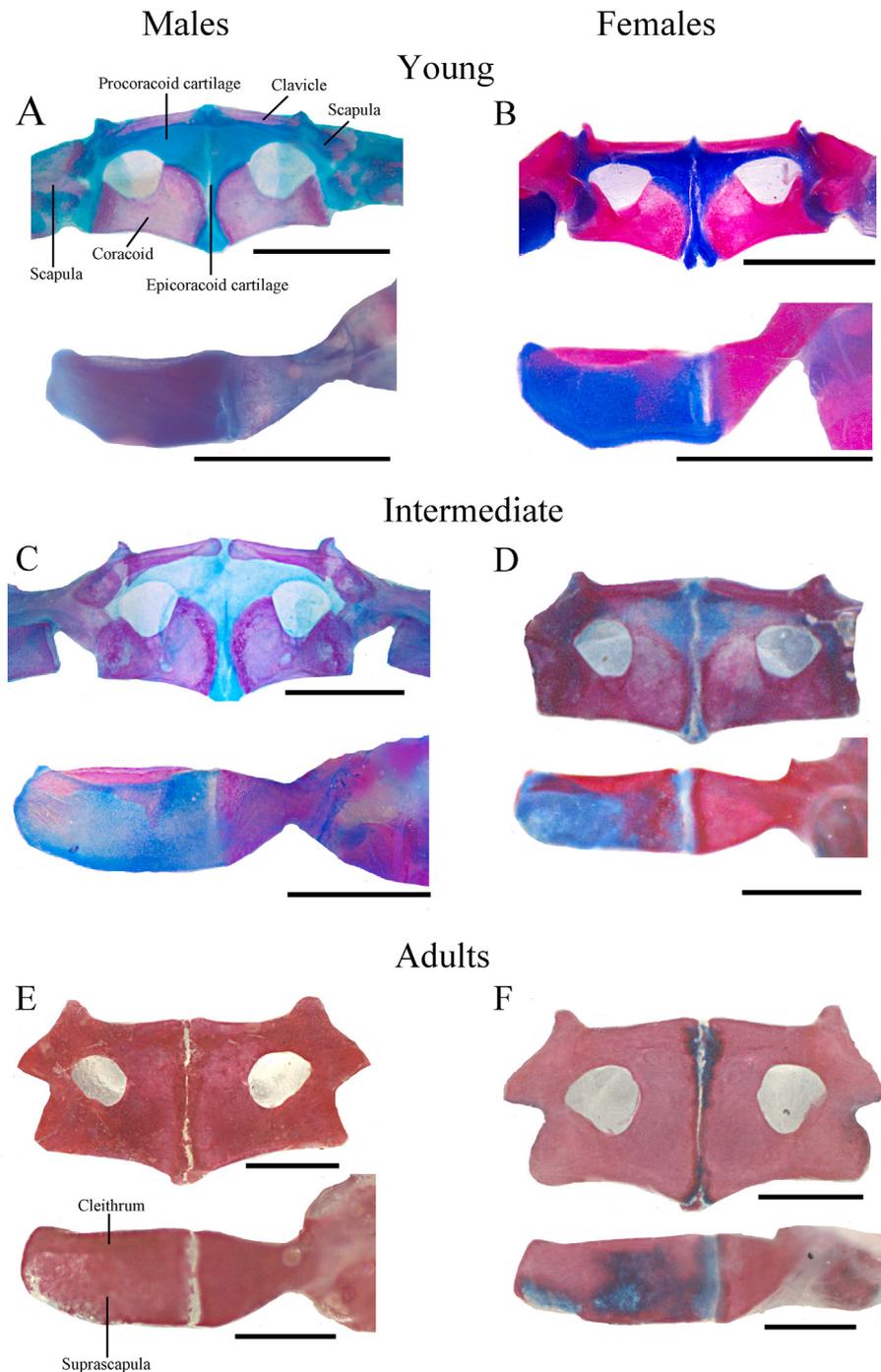


Fig. 5. Ontogenetic post-hatching development of the pectoral girdle of *Brachycephalus garbeanus*. (A) young male (MNRJ 39611; SVL 9.8 mm); (B) young female (MNRJ 93599; SVL 10.1 mm); (C) intermediate male (MNRJ 92805; SVL 10.9mm); (D) intermediate female (MNRJ 39612; SVL 12.2mm); (E) adult male (MNRJ 92803; SVL 14.8 mm); and, (F) adult female (MNRJ 93602; SVL 18.3 mm). Scale bar = 1 mm.

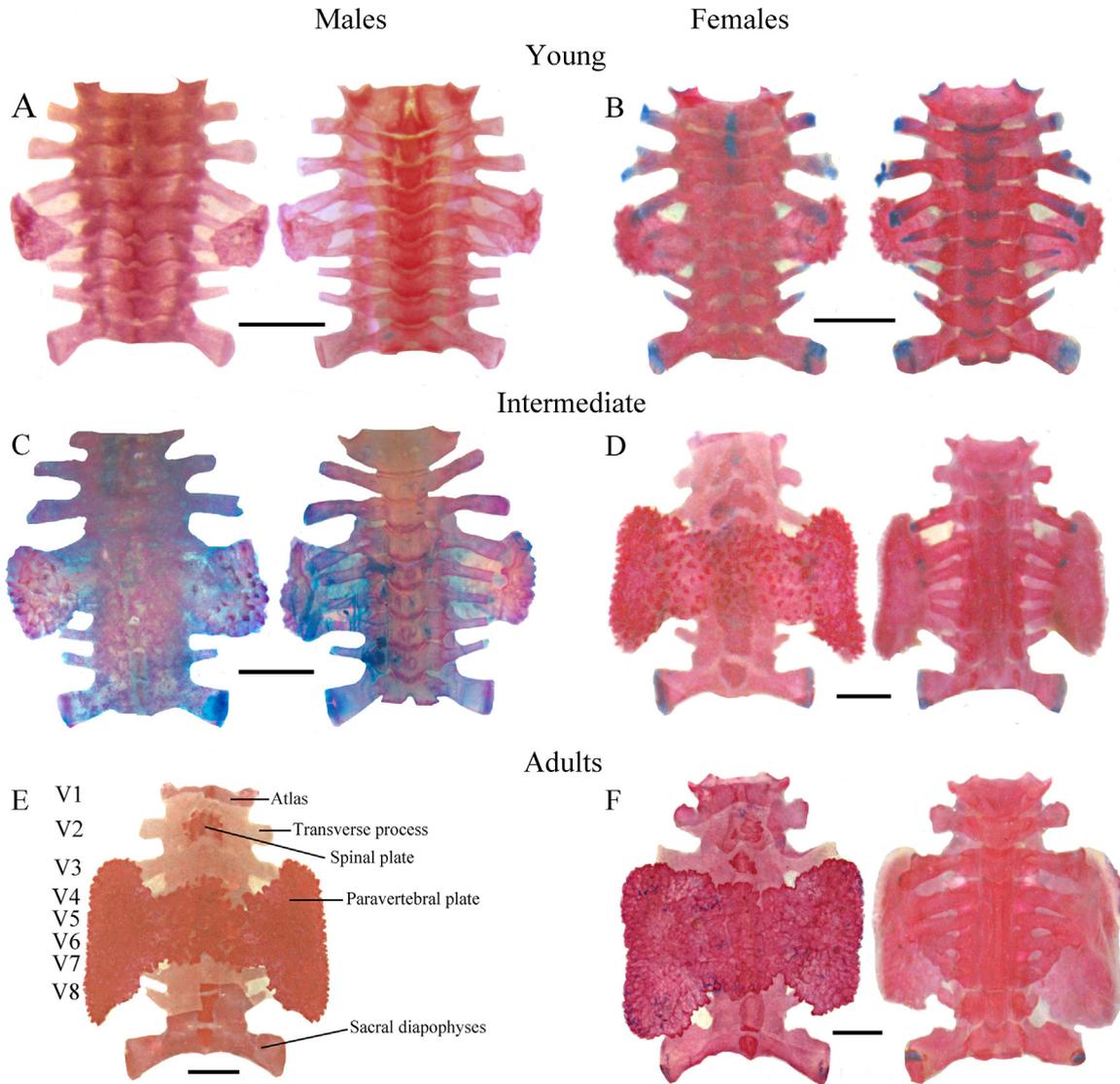


Fig. 6. Ontogenetic post-hatching development of the vertebral column (dorsal and ventral views) of *Brachycephalus garbeanus*. (A) young male (MNRJ 92792; SVL 8.9 mm); (B) young female (MNRJ 93599; SVL 10.1 mm); (C) intermediate male (MNRJ 92805; SVL 10.9 mm); (D) intermediate female (MNRJ 93600; SVL 15.4 mm); (E) adult male (MNRJ 92803; SVL 14.8 mm); and, (F) adult female (MNRJ 93602; SVL 18.3 mm). Scale bar = 1 mm.

dorsum, being variable in shape and located at each side of the main axis of the vertebral column. The paravertebral plates are small trapezoidal structures that cover only the transverse processes of vertebrae IV, V and VI, and are isolated from the vertebrae and vertebral processes. Later, the paravertebral plates and the aforementioned transverse processes are fused. The urostyle is well-ossified, long, slender, and does not reach the interiliac suture. Young and intermediate females exhibit an inconspicuous distal cartilaginous tip at this element. The urostyle exhibits a dorsal crest, with an anterior dilatation that extends throughout its whole extension. Its height at its anterior end is about four times wider than the posterior one, progressively decreasing in height anteroposteriorly.

3.3.2. Pectoral girdle (Fig. 5)

Young specimens have a thin, fully ossified and straight clavicle. The coracoid is broad, enlarged at its proximal margin, with thinner medial margins when compared to the lateral ones. The scapula,

procoracoid and epicoracoid are not ossified at this stage (Fig. 5A and B). The suprascapula is not expanded, and its anterior half is ossified with the cleithrum. Intermediate males have slightly enlarged clavicles and cartilaginous procoracoids, epicoracoids and suprascapula (Fig. 5C). Intermediate females have an enlarged clavicle and an almost fully ossified procoracoid; in these individuals, the epicoracoid is almost fully ossified. Also, the proximal end of suprascapula is mineralized (Fig. 5D). Both intermediate males and females have almost fully ossified scapulas. Adult specimens have a completely ossified and fused clavicle, procoracoid, epicoracoid, coracoid, and scapula, with a wide oval fenestra between the procoracoids and coracoid (Fig. 5E and F). The omosternum and sternum are absent. The coracoid is deeply concave anteriorly, with both medial and lateral portions well-developed and expanded. The distal edge of the clavicle bears an anterior projection. Adult males have fully mineralized suprascapulas, while in females this bone is slightly less mineralized.

3.3.3. Pelvic girdle (Fig. 6)

All elements are fully ossified in all stages, with no morphological variation between individuals. The sacral-coccygeal articulation is bicondylar, and the pelvic plate is semicircular. The angle between the ilial shaft and preacetabular expansion is 90° , with the ventral edge of the ischium surpassing the level of the ilium. The ilial shaft is rectangular in cross-section related to its dorsal crest. The pubis represents a mineralized wedge located between the ilium and ischium. The dorsal crest is normally developed (height equal to the dorsal acetabular expansion), gradually increasing in height from the point where it articulates with the sacral diapophysis.

3.3.4. Forelimb (Fig. 7)

The humerus bears a well-developed ventral crest that extends from the glenoidal head until reaching half the bone length. A posterior and inconspicuous crest is also present throughout almost the total length of the bone. The glenoidal head of the humerus is slightly broader than the distal head, and both heads are rounded, compressed, and totally ossified. The radioulna is flattened, with the sulcus intermedius being evident and extending from the distal head to about the element midlength. There is no conspicuous variation between sexes or stages in the individuals examined herein. *Manus* (Fig. 7)-Young and intermediate individuals have the same distinguishable elements found in adults, except for the presence of sesamoids, which are present only in intermediate males and adult females. In young individuals, the metacarpal and phalangeal elements are essentially ossified along their main body, but cartilaginous at their distal tips; the carpal elements are totally cartilaginous (Fig. 7A and B). In intermediate males, the carpal elements remain cartilaginous, but in females are partially ossified. Intermediate females lack palmar sesamoids. Full ossification of manus elements occurs in adults, although the carpal elements remain partially cartilaginous in two adult females (SVL 18.3 mm and 17.3 mm). The first phalanx of fingers II and V only fully ossify in adults.

Adult specimens have four carpal elements consistent with Fabrezi's (1992) Type E morphology—i.e., ulnare; Distal Carpal (DC) 5–4–3; DC 2 with y-element; and radiale and proximal elements of the prepollex. The largest carpal bone is DC 5–4–3, which articulates with Metacarpals III–V, and the fused y-element with DC 2 articulating with Metacarpal II and prepollex, which is composed of two ossified and greatly reduced elements. The relative length of carpal elements is Carpal 3–4–5 > ulnare = radiale > DC 2 with a y-element. The phalangeal formula is 1–2–3–1, the relative digit lengths being IV < III < V = II. Tips of the terminal phalangeal elements of fingers (except last phalanx) are arrow shaped. A palmar sesamoid is present in ventral view.

3.3.5. Hindlimb (Fig. 8)

The femur is about the same length as the tibiofibula, whereas the tibiale-fibulare is slightly shorter than the latter. In young and intermediate specimens (males and females), the proximal and distal heads of these elements are partially cartilaginous, while in adults they are totally ossified. A distinct sulcus intermedius is present on both sides of this element. The tibiofibular are fused at their proximal and distal extremities but medially separated at their midlength. Mineralized, spherical sesamoids are present in the femur-tibiofibula articulation, but are only seen in young males, intermediate females, and adults (both males and females) – the larger one most likely represents the graciella sesamoid. The size of the sesamoids relatively increases from young to adults. An additional sesamoid is found at the tibiofibular-tibiale/fibulare articulation, being ossified and first seen even in the early stages of young individuals. *Pes* (Fig. 8)-Young and intermediate individuals have –

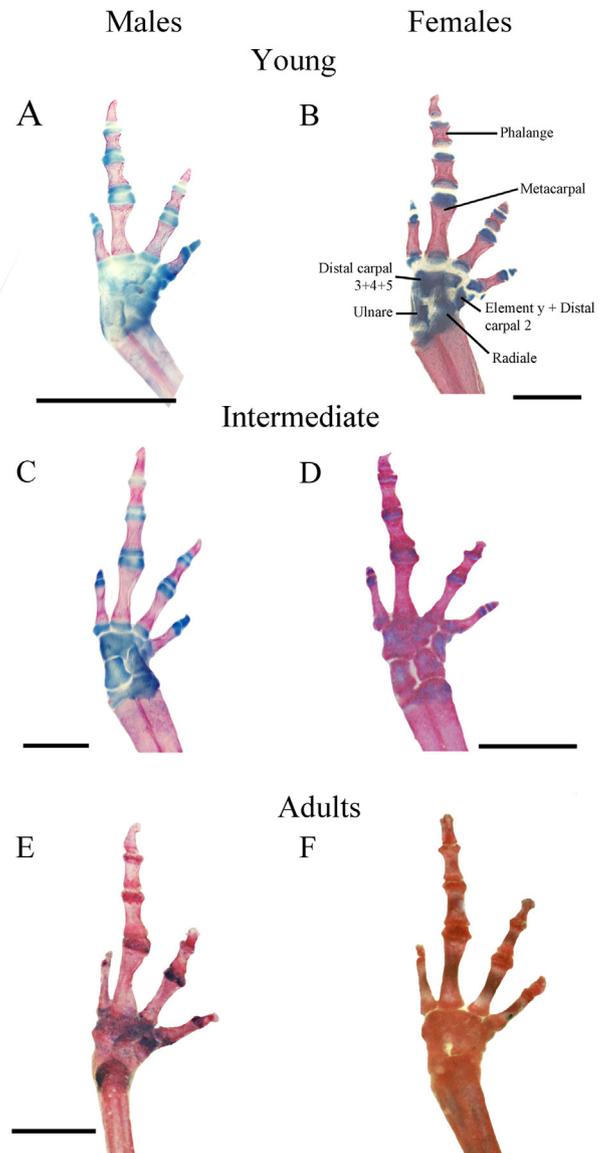


Fig. 7. Ontogenetic post-hatching development of the *manus* of *Brachycephalus garbeanus*. (A) young male (MNRJ 39611; SVL 9.8 mm); (B) young female (MNRJ 93599; SVL 10.1 mm); (C) intermediate male (MNRJ 92805; SVL 10.9 mm); (D) intermediate female (MNRJ 39612; SVL 12.2 mm); (E) adult male (MNRJ 92803; SVL 14.8 mm); and, (F) adult female (MNRJ 93602; SVL 18.3 mm). Scale bar = 1 mm.

except for the presence/absence of sesamoids – the same elements as found in the adults. However, the phalanges and metatarsal elements have conspicuous cartilaginous tips that gradually ossify from young to adults. The tarsal elements are totally cartilaginous in young and intermediate males, while in young females these elements are already partially ossified, and almost fully ossified in intermediate ones. Full ossification of tarsal elements is found in both male and female adults, although in a few specimens these elements are still not fully ossified. Cartilago sesamoids are present, and a plantar sesamoid is present in intermediate females, and adult males. Tarsus of adult specimens are composed of tibiale, fibulare, three individual elements, including Distal Tarsal (DT) 2–3, DT 1, y-element. DT 1 is the smallest and articulates with y-element, distal tarsal 2–3, and metatarsal I and II. DT 2–3 articulates mainly with metatarsal III, also with metatarsal II and IV, and with distal tarsal I. The relative length of tarsal elements is DT 2–3 > y-element > DT 1. The phalangeal formula is 1–2–3–4–1,

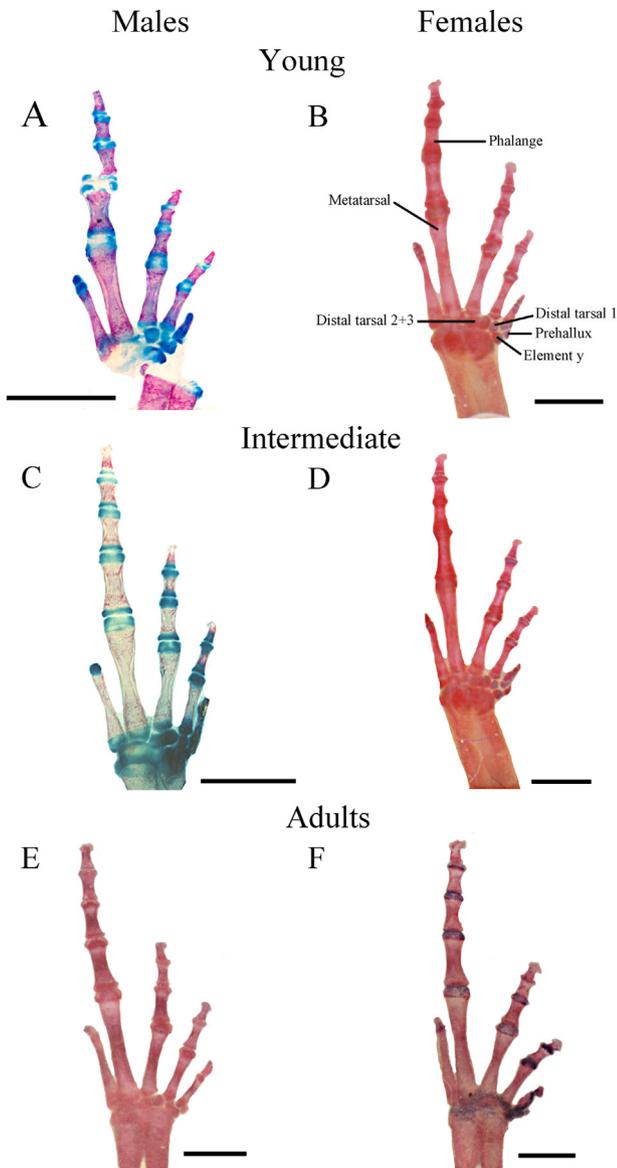


Fig. 8. Ontogenetic post-hatching development of the pes of *Brachycephalus garbeanus*. (A) young male (MNRJ 39611; SVL 9.8 mm); (B) young female (MNRJ 93599; SVL 10.1 mm); (C) intermediate male (MNRJ 92805; SVL 10.9 mm); (D) intermediate female (MNRJ 93600; SVL 15.4 mm); (E) adult male (MNRJ 92803; SVL 14.8 mm); and, (F) adult female (MNRJ 93602; SVL 18.3 mm). Scale bar = 1 mm.

the relative digit lengths being: $IV < III < V < II < I$. The prehallux has one calcified element. The tips of the terminal phalangeal elements of toes II–IV are arrow-shaped. Toes I and V are reduced with tips of terminal phalangeal elements pointed, being elongated on digit IV.

4. Discussion

4.1. Osteology and ontogenetic variation of the skeleton of *Brachycephalus garbeanus*

Herein we provide the first detailed description of the post-hatching osteological development of *B. garbeanus*. Even though a few taxa from the genus have previously been — at different levels and with several purposes — investigated regarding its osteology (e.g., Campos et al. 2010; Vera Candiotti et al. 2020; Trueb & Alberch 1985), our results allow us to discuss several developmental

patterns for the species and its morphology in terms of the extreme taxa miniaturization.

Studies on the ontogenetic development of brachycephaloids reveal interesting patterns of fundamental repatterning of cartilaginous scaffolds and ossification sequences during ontogenetic development, as well as the loss and appearance of several structures (Vera Candiotti et al. 2020; Hanken 1992). Previous evidence shows that ossification rates in the prehatching stages of *B. ephippium* are comparatively delayed in comparison to other brachycephaloid taxa as *Ischnocnema*, *Oreobates*, and *Haddadus* (Vera Candiotti et al. 2020), with a few structures — as the palatoquadrate — not reaching its adult arrangement before hatching. The results found herein for the posthatching osteology of *B. garbeanus* seem to corroborate such findings, given that several skull, axial and appendicular structures are not fully developed until the specimens reach larger sizes (= later developmental stages, herein called “adults”). For instance, the hyperossification and bone fusion of the parotic plate, dorsal skull, and pectoral girdle elements are only fully attained at later post-hatching stages (Campos et al., 2010 present study).

Most of the ontogenetic variation in the vertebral column of *B. garbeanus* is associated with the level of ossification and ornamentation of the spinal and paravertebral plates, which in later stages are expanded to fuse into a dorsal and highly ornamented bony shield. The paravertebral plates are lateral and reduced in young specimens, emerging at early stages and without the connection to any other axial element, in a similar pattern previously found for other *Brachycephalus* (Campos et al. 2010). Therefore, our results reinforce that the paravertebral plate is most likely a neomorph and does not represent a hyperossification of any axial element (Campos et al. 2010). Additionally, the spinal plates are intimately associated with the dorsal surface of the vertebrae, enlarging at later stages. However, even though our results might lead to the assumption that these plates represent vertebral hyperossifications, previous histological studies (Campos et al. 2010) report that such a structure is formed by the addition of intramembranous bone tissue. In such a view, additional histological studies are needed to corroborate the nature of such bony elements in *Brachycephalus* species (except for *Brachycephalus ephippium*).

Posthatching developmental data herein show that there is no conspicuous sexual dimorphism in the ontogenetic trajectory of males and females from the stages provided herein. However, the stages categorized herein considered females to be larger than males (but see Table 1), and consequently this shows that males attain bone ossification at smaller sizes in comparison with females. Even though our samples were satisfactory for discussing ontogenetic trajectories of bone ossification, additional samples are needed in order to understand detailed sexual dimorphic trajectories related to bone degree of ossification. Previous studies regarding sexual dimorphism in the degree of ossification of indirect-development anurans during their ontogenetic trajectories are inconstant, as they might indicate the presence of variation between sexes (e.g. Barrionuevo, 2013; Arantes et al., 2015) or, on the contrary, reveal no direct evidence of variation during the ontogeny of taxa (e.g. Ponssa, 2008; Ponssa and Barrionuevo, 2010). Thus, further investigation with a broader sample might elucidate whether the presence of this phenomenon is present in *Brachycephalus*.

4.2. Miniaturization and the “morphological contradiction” of *Brachycephalus*

The phenomenon of miniaturization has evolved numerous times amongst vertebrates and invertebrates and has reached

impressive extremes in anurans (Britz & Kottelat 2008; Glaw et al. 2021; Hanken 1984, 1983; Hanken & Wake 1993; Yeh, 2002). Rather than merely attaining a very reduced size, miniaturization is frequently associated with three phenotypic consequences: (i) emergence of morphological novelties, (ii) structural simplification, and (iii) increased intraspecific variation (Hanken & Wake 1993).

Miniaturization itself has previously been considered as one of the main constraints for the evolution of direct development amongst anurans (Rittmeyer et al. 2012), and available developmental studies for miniaturized frogs have frequently evidenced heterochronic changes as a major driving mechanism for the emergence of novelties and structural simplification in such reduced body sizes (Davies 1989; Fabrezi 2011; Trueb & Alberch 1985). In such a scenario, paedomorphosis and peramorphosis have been associated with the miniaturized phenotypic consequences in these lineages (Gould 1977; Hanken & Wake 1993). While paedomorphosis occurs when later stages of ontogeny retain characteristics of earlier stages of the ancestor development, peramorphosis associated with the extension of the development beyond the stages of its ancestral lineage, generating new or even hypertrophied structures (Hanken 1984; Hanken & Wake 1993; Klingenberg 1998; Paluh et al. 2020; Yeh, 2002).

The miniaturized genus *Brachycephalus* has previously been considered as a “morphological contradiction” (see Trueb & Alberch 1985) due to the combination of paedomorphic and peramorphic features possibly related to miniaturization. Indeed, a few morphological characteristics found for *B. garbeanus* might be attained by paedomorphosis and peramorphosis, reinforcing previous findings for other congeners. Most of the morphological novelties found in the species are related to the dorsal skull elements and the emergence of the parotic and paravertebral plates; while morphological simplification is found in several elements of the skull and appendicular skeleton, such as the fusion of appendicular elements and digit loss (as found in several vertebrate lineages; Hanken & Wake 1993; Rieppel 1996; Yeh, 2002).

Phylogenetic bone loss due to developmental truncation has received considerable attention in previous studies of vertebrate miniaturization, with such a loss being extensively reported in miniaturized anurans (e.g., Hanken & Wake 1993; Trueb & Alberch 1985; Yeh, 2002). Skull bones that are frequently lost in miniaturized in anurans — palatines, columellae, and quadratojugals — are among the last to ossify in anurans, exhibiting greater disparity and faster evolution rates (Bardua et al. 2021; Trueb & Alberch 1985), being consistent with paedomorphic. For instance, the columellae seem to be absent in *Brachycephalus*, and the neopalatines and quadratojugals are reduced or completely lost in a few species (see Folly et al. 2020; present study). Furthermore, the genus is known to have lost a series of phalanges in both *manus* and *pes* (Da Silva et al. 2007). *B. garbeanus* exhibits a *manus* phalangeal formula 1–2–3–1 and *pes* 1–2–3–4–1, reinforcing the relative reduction of phalangeal elements within the genus. In addition, Vera Candioti et al. (2020) interpret the independent y-element as a paedomorphic state for *B. ephippium*.

Function and sound transmission in frogs might also be constrained by the extreme reduction of the auditory apparatus, and the loss of structures might also occur in miniaturized species (Da Silva et al. 2007; Duellman & Trueb 1994). Our results show the complete absence of the columellae in *B. garbeanus* and the presence of a wide and well-developed operculum in all specimens. According to previous studies, truncation of development at early development stages might be associated with the simplified morphology of this system, as well as with the origin of a novel mechanism of sound transmission (Da Silva et al. 2007; Yeh, 2002). Additionally, an enlarged operculum and a posteriorly oriented fenestra ovalis have previously been hypothesized as putative

synapomorphy for the genus (Da Silva et al. 2007), and both hypotheses — novel sound transmission mechanism and synapomorphy — still need to be tested.

Structural simplification in anurans also tends to manifest as the fusion of several skull bones (Yeh, 2002). For instance, in a few *Brachycephalus* (including *B. garbeanus*) the vomers are co-ossified with the sphenethmoid (Bornschein et al. 2016; Ribeiro et al. 2017). This type of simplification is also found in a few post-cranial elements of *Brachycephalus* spp., such as the fusion of appendicular elements and vertebrae (Folly et al. 2020; present study). For skull bones, fusion is known to occur mainly because there is a minimum critical size in which the braincase can reduce, and thus simplification is attained by fusion to maintain a relatively large neurocranium (Paluh et al. 2020; Rieppel 1996).

The general anatomy of the pectoral girdle seems to have a strong phylogenetic signal amongst anurans, with a reduced effect of the type of locomotion on the morphology of the components of this region (Emerson 1983; Engelkes et al. 2020). Bone fusion in the elements of the pectoral bones in *Brachycephalus* are unique amongst anurans. Indeed, the synosteoal union of the procoracoid with clavicle and scapula has already been pointed as a unique specialization of the genus, and the ossification of the pectoral bones might be associated with a higher biomechanical demand imposed by the need for insertion for larger muscles and/or larger muscle insertion sites, and, since these elements remain cartilaginous in most anuran adults (Trueb 1973), its ossification might be associated with the compensation for its extremely miniaturized size.

Extreme size decrease is usually associated with the emergence of several morphological novelties, which seem to compensate for the reduction of organs and/or systems of vital function (Hanken & Wake 1993). Most of such novelties have been described for the skeletal system of several vertebrates, with hyperossified skeletal elements — both skull and post-cranium — being reported as a result of miniaturization (Yeh, 2002). Although skull hyperossification has previously been hypothesized as a byproduct of miniaturization in anurans, such a view has been rejected for this group as a whole (Paluh et al. 2020). Recent data suggests that hyperossification is not exclusively related to miniaturized anurans, and most likely represents a peramorphic trait possibly associated with accelerated ontogenetic trajectories and morphological change unrelated to size reduction (Paluh et al. 2020). Campos et al. (2010) have found that the parotic and spinal plates are formed by intramembranous bone tissue addition, and therefore do not represent hyperossified structures, but most likely additional structures that have arisen in these lineages. Therefore, developmental, and histological data seem to indicate that true hyperossification might only be present between and among vertebrae, generating the typical fusion of presacral vertebrae III to VII.

During the course of anuran evolution, the hypossification of elements from the pectoral girdle has been historically associated with paedomorphosis (Havelková and Roček, 2006). In *Brachycephalus*, however, while some elements seem to be developmentally arrested and completely lost (e.g., omosternum and sternum), the procoracoid, coracoid, epicoracoid, clavicle, and scapula represent a single ossified element. Since the omosternum represents a neomorph that helps to cushion the shock of landing in jumping frogs (Havelková and Roček, 2006), it seems plausible to hypothesize that the loss of such an element in *Brachycephalus* might have been favored by its locomotion mode — i.e. walking rather than jumping (e.g. Folly et al., 2021a, 2021b; Pombal et al., 1994)— also facilitating extreme size reduction. The cartilaginous condition of elements are only present in earlier stages, with a high degree of ossification and bone fusion in later stages. Such a condition — i.e. bone fusion in adults — might have been attained by peramorphosis and must be

further investigated. Indeed, if such a hypothesis is confirmed, the 'morphological contradiction' is again present in the pectoral girdle, with paedomorphosis arresting the development of a few structures, while peramorphosis occurs in both endochondral and dermal bones to generate a morphological novelty.

The third morphological consequence associated with miniaturization refers to increased intraspecific variation (Hanken & Wake 1993). Even though such a consequence has been historically more investigated and reported for amphibians (Hanken & Wake 1993), recent studies with higher samples have also reported the presence of such a phenomenon in other vertebrates (e.g., Martins et al. 2021). Even though our samples for adult specimens were small, except for general skull shape – which might be associated with allometric variation – the only striking intraspecific variation between the adult specimens of *B. garbeanus* was the ossification and presence of sacral diapophysis sesamoids. Since the process of ossification/chondrification of sesamoids is under both genetic and epigenetic control (Abdala et al. 2019; Sarin & Carter 2000), and that embryonic movement represents a key aspect in the genesis of sesamoids (Abdala et al. 2019), the dimorphic variation found herein might be explained considering that the presence/absence of the sesamoids vary at an intrapopulation level, with environmental and embryonic forces acting on their differentiation. For instance, its presence might be biochemically controlled since these structures are fully ossified in larger individuals. Considering these elements only appear in adult males; and in females they might be fully ossified in intermediate individuals, these sesamoids are most likely present for physical resistance such in larger-sized individuals.

4.3. Limitations and future perspectives

Our study represents one of the many first steps on the understanding of osteological development of direct-development anurans. We acknowledge that the existence of earlier post-hatching stages would have allowed us to understand the exact time of the emergence of several morphological novelties – as the parotic plate – as well as having a better approximation of the exact timing of ossification of several bones described herein. However, our sample was satisfactory for evaluating that the skeleton of *B. garbeanus* exhibits great variation through an ontogenetic trajectory and – when compared with other available studies – allows us to corroborate the hypothesis of its delayed developmental osteology in comparison with other brachycephaloids. We encourage that future studies aim not merely at describing the full skeleton of direct-developing anurans, but also their variation in terms of pre- and post-hatching ontogeny. As in direct-developing anurans the constraints in the cranium and suspensorium morphology are more relaxed (Bardua et al. 2021), additional developmental studies will allow – in association with recent phylogenetic hypotheses – to understand the patterns and processes related to the existing phenotypic variation. These comparative data will also allow robust interpretations of the morphological consequences of miniaturization in the genus, evaluating whether the morphology is solely based on heterochronic changes or as a result of extreme miniaturization.

Herein we made an approximation of developmental stages based on the size of individuals from our sample, as well as on Campos et al. (2010), and thus these stages, unfortunately, do not rely on gonadal maturity data. Further studies on gonad differentiation and the timing of sexual maturity in *Brachycephalus* will allow not only the selection of more accurate post-hatching developmental categories, as the understanding of the association of skeletal changes related to those modifications.

Finally, additional studies will also be decisive in understanding the homology of several elements and their relation to the retention of paedomorphic features. As miniaturized species usually lack several elements, understanding the development of the remaining bones and how they change over time are also relevant in understanding the developmental adjustments that occur especially in lineages where a larval stage is absent.

5. Conclusion

The description of the post-hatching skeletal development in *B. garbeanus* allows us to assume that the ossification of several skull, appendicular and axial elements are delayed and only occur at later stages when individuals reach >12 mm of SVL. Earlier post-hatching stages (~8–10 mm) are characterized by a series of non-ossified elements in the appendicular skeleton and unfused elements of the skull roof and vertebrae. While during development the ossification of elements between males and females seems to be desynchronized, later stages and final stages (i.e. > 14 for males and >17 for females) do not exhibit conspicuous dimorphic variation. As there is evident post-hatching ontogenetic variation in *Brachycephalus*, future osteological data must acknowledge different developmental stages, especially those using such data for systematic purposes.

Finally, several osteological simplifications of the genus – as the reduction and loss of phalangeal elements and bone fusion – might have emerged to compensate the extremely miniaturized body of the lineage. On the other hand, skull and axial osseous plates/shields must be investigated for a histological and developmental purpose to understand the nature of these elements, for instance (i) if such novelties are due to hyperossification of structures over the developmental time (i.e. peramorphosis); and/or (ii) if they represent novel structures that arose independently in a few taxa of the lineage.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jcz.2022.02.005>.

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