

Development of the bony skeleton in the Taiwan salamander, *Hynobius formosanus* MAKI, 1922 (Caudata: Hynobiidae): Heterochronies and reductions

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Abstract

The development of the bony skeleton in a partially embryonized lotic-breeding salamander *Hynobius formosanus* is studied using the ontogenetic series from late embryos to postmetamorphic juveniles and adult specimen. Early stages of skull development in this species are compared with the early cranial ontogeny in two non-embryonized lentic-breeding species *H. lichenatus* and *H. nigrescens*. The obtained results show that skeletal development distinguishes *H. formosanus* from other hynobiids by a set of important features: 1) the reduction of provisory ossifications (complete absence of palatine and reduced state of coronoid), 2) alteration of a typical sequence of ossification appearance, namely, the delayed formation of vomer and coronoid, and 3) the absence of a separate ossification center of a lacrimal and formation of a single prefrontolacrimal. These unique osteological characters in *H. formosanus* are admittedly connected with specific traits of its life history, including partial embryonization, endogenous feeding until the end of metamorphosis and relatively short larval period.

Key words

Hynobiid salamanders, osteology, cranial ontogeny, embryonization, endogenous feeding, evolution.

Introduction

The development of the skeleton, especially the skull, in urodelan amphibians is strongly affected by the life history. Thus, it is widely known that paedomorphic salamanders which retain larval morphology in the adult state do not complete metamorphic remodeling of the skull. They display diverse juvenile traits in their cranial bone composition and arrangement, as well as in their dentition and hyobranchium; the latest stages of skeletal development are usually omitted in these urodelans (see ROSE, 2003 for review; IVANOVIĆ *et al.*, 2014). In contrast, some features of early skeletal ontogeny are omitted in the direct-developing lungless salamanders

(Plethodontidae) lacking larval period (WAKE, 1966; ALBERCH, 1987; WAKE & HANKEN, 1996; EHMCKE & CLEMEN, 2000; MARKS, 2000; DULCEY CALA *et al.*, 2009). The influence of life history on skull development is studied in more detail in the plethodontids which embrace paedomorphic, metamorphosing and highly embryonized direct-developing species (WAKE, 1966; MUTZ & CLEMEN, 1992; ROSE, 1995; 1996; MARKS, 2000). Although direct developers are not encountered among other urodelan families, many salamander species display a certain degree of the embryonization of their ontogeny. Embryonization implies the incorporation of larval stag-

es into the intraoval development (MATSUDA, 1987) and may lead to the direct development without free larval stage. Caudate amphibians with partial embryonation have relatively larger eggs with abundant yolk reserves, longer embryonic period and their larvae hatch at more advanced stages compared with non-embryonized species. Apart from some plethodontids, partially embryonized ontogeny is characteristic, for example, for *Amphiura means* (Amphiumidae) (GUNZBURGER, 2003), *Rhyacotriton* spp. (Rhyacotritonidae) (WORTHINGTON & WAKE, 1971; KARRAKER, 1999), *Chioglossa lusitanica* and *Mertensiella caucasica* (Salamandridae) (TARKHNI-SHVILI & SERBINOVA, 1993; 1997; SEQUEIRA *et al.*, 2003). The Hynobiidae also include species, which fit certain criteria of embryonation, for example, the Asian clawed salamanders *Onychodactylus* spp. (REGEL & EPSTEIN, 1975; IWASAWA & KERA, 1980; AKITA & MIYAZAKI, 1991; SERBINOVA & SOLKIN, 1992; PARK, 2005; POYARKOV *et al.*, 2012) and some lotic-breeding species of the genus *Hynobius*, for example *H. kimurae* (MISAWA & MATSUI, 1997a; AKITA, 2001) and especially *H. formosanus* and *H. sonani* inhabiting the mountain streams in the highlands of Taiwan (KAKEGAWA *et al.*, 1989). Previous studies revealed that skeletal ontogeny in embryonized hynobiids displays, in particular, certain heterochronic shifts in the development of the skull bones and the reduction of those bones which normally develop in larvae and disappear during metamorphosis (MISAWA & MATSUI, 1997b; SMIRNOV & VASSILIEVA, 2002; VASSILIEVA *et al.*, 2013). Among other embryonized *Hynobius* species the Taiwan salamander *H. formosanus* is of special interest for the morphological study because this species displays certain unique life history traits. Namely, *H. formosanus* has a remarkably short larval period (LUE *et al.*, 1982; KAKEGAWA *et al.*, 1989; LUE & CHUANG, 1992) and is the only hynobiid known to date which may complete the metamorphosis relying only on its massive yolk reserves without exogenous feeding (KAKEGAWA *et al.*, 1989; our data). Some preliminary data on *H. formosanus* development reported by us (CHANG *et al.*, 2009) indicate that the specific life history traits in this species apparently affect its cranial ontogeny. Herein we present the detailed description of the skeletal development in *H. formosanus* and compare it with available data on the development in other hynobiids.

Materials and Methods

Skeletal morphology and development of *Hynobius formosanus* were examined in an ontogenetic series of late embryos, larvae, and postmetamorphic individuals obtained from the eggs collected in the natural breeding sites. The clutches (egg sacs) were collected in two localities in central Taiwan. In January and February 2008, *H. formosanus* reproduction was observed by Nikolay A. Poyarkov and Yu-Hao Chang in road-side ditches made of stones and filled with water from small springs and

streams along the road from Lushan to Songyuan and Tienchi mountain shelter on the slopes of Nenggao-Shan Mt., Renai Township, Nantou County, Taiwan (24.0471° N; 121.2564° E; 2576 m a.s.l., egg sacs collected on 13 February 2008). The other group of clutches was collected on 15 June 2009 by Nikolay A. Poyarkov, Yu-Hao Chang, Yong-Cheng Huang and Li-Yi Chang in a small intermittent mountain stream on the slopes of Pintian-Shan near the mountain trail from Wuling to Shyue-Shan in Jianshi Township, Hsinchu County, Taiwan (24.4324° N; 121.2754° E; 3130 m a.s.l.). After collection and transportation, the egg sacs were placed under laboratory conditions in Moscow State University (2008) and in National Taiwan Normal University (2009). Eggs in all egg sacs collected were counted, diameters of eggs at the earliest stages of cleavage (zygote / two blastomeres) were measured with a digital caliper to the nearest 0.01 mm. In total, 12 and 7 clutches were collected in 2008 and 2009, respectively. Embryos and larvae were reared in the aquaria with conditioned highly aerated tap water at the temperature 10 °C (NTNU laboratory) or 14–16 °C (MSU laboratory) until the completion of metamorphosis. Metamorphosed juveniles from the clutches obtained in 2009 were kept for one year in plastic containers (15 × 10 cm) with wet sponges and shelters on the bottom and fed *ad libitum* with *Drosophila* sp. (NTNU laboratory). An adult specimen of *H. formosanus* (female, TWN-09 H-005) used in the study was collected in the same site as the clutches in February 2009 and preserved in 75 % ethanol. The late embryos (with differentiated limbs), larvae, and postmetamorphic juveniles were regularly euthanized at different stages and fixed in 10% neutral-buffered formalin. In total, 50 specimens of *H. formosanus* were used in the present study.

Additionally, for comparison, we examined series of early larvae of two lentic *Hynobius* species, the Tohoku salamander *H. lichenatus* and the Japanese black salamander *H. nigrescens* (11 and 8 specimens, respectively), kindly provided by Dr. Tadashi Nakazato (Nakadai Junior High School, Tokyo, Japan). The egg sacs of both species were collected in a small natural pond on slopes of Fuji-san Mt., Yumotoshiobara, Tochigi Prefecture (36.9555° N; 139.7871° E; 1120 m a.s.l.). After hatching, larvae were reared in the laboratory of Nakadai Junior High School (Itabashi, Itabashi-ku, Tokyo) in plastic containers 15 × 10 cm filled with aerated tap water until several posthatching days and preserved in 75% ethanol prior to the beginning of exogenous feeding.

In all specimens, the snout-vent length (SVL) and total length (TL) were measured using a digital caliper to the nearest 0.1 mm; all numeric parameters are given as mean ± SD. To facilitate the interspecific comparison of the developmental features, *H. formosanus* embryos and larvae were staged according to the tables of normal development of both *Onychodactylus japonicus* (IWASAWA & KERA, 1980) and *Hynobius nigrescens* (IWASAWA & YAMASHITA, 1991), omitting features connected with the development of V toe, claws, and balancers. In the chapter “Results” stages of *H. formosanus* are given in a

Table 1. Specimens of *Hynobius* species used for the study of skeleton development.

Stage after IWASAWA & KERA (1980)	Stage after IWASAWA & YAMASHITA (1991)	SVL (mm)	TL (mm)	Number of specimens
<i>Hynobius formosanus</i>				
48–49	42–43	9.0	14.0	1
50	43	9.5±0.5	14.4±0.1	2
51	44	9.5±0.9	13.5±0.2	2
52	46	9.7±0.9	14.7±0.8	5
53	48	10.1±1.1	15.9±1.3	6
54–55	50–52	11.4±1.3	18.1±2.3	7
56–57	53–54	10.9	17.6	1
58	54–55	9.8	17.9	1
60–61	57–58	14.1	25.2	1
62–63	58	12.2±0.1	20.1±2.8	2
68	63B	14.0±0.4	23.7±1.5	2
69–70	64	13.9	21.8	1
71–72	65–68	15.0±0.6	24.5±1.3	6
Postmetamorphic juveniles		13.4±0.8	21.6±2.0	12
Adult		52.5	103.6	1
<i>Hynobius lichenatus</i>				
	40–41	9.3±0.4	13.0	2
	42–43	10.2±0.3	17.3±0.6	3
	46	10.5±0.5	17.7±0.6	3
	47	11.5±0.5	18.6±0.5	3
<i>Hynobius nigrescens</i>				
	36	9.1	11.5	1
	40–41	8.1±0.3	12.6±0.9	4
	42–43	8.8±1.0	14.8±1.7	2
	46	10.2	17.3	1

double mode I&K [I&Y]. Larvae of *H. lichenatus* and *H. nigrescens* were staged after IWASAWA & YAMASHITA, 1991. The composition of the studied *Hynobius* spp. developmental series is given in Table 1.

Prior to examination, all specimens were prepared as skeletal whole-mounts by staining with Alizarin red for calcium deposits and cleared in 1% KOH. Nine specimens of *H. formosanus* (stages 52, 53, 54–55, 58) were double-stained for bone and cartilage with Alizarin and Alcian blue, respectively. We followed the terminology of ROSE (2003) for the designation of bones and cartilages of the skull and hyobranchial apparatus.

The development of ossifications was examined using a LEICA EZ4 binocular dissecting microscope with a digital camera. Original drawings were made by A.B. Vassilieva. All specimens used in the present study are deposited in the Zoological Museum of Lomonosov Moscow State University (ZMMU).

Results

Eggs, clutches, and general features of the development. Each clutch represents a pair of elongated, spirally curved eggs sacs with transparent gelatinous, slightly opalescent walls and large white eggs inside (Fig. 1A).

Clutch size varies from 13 to 28 eggs (17.1 ± 4.8 , $n=12$, with 8.5 ± 2.5 egg per egg sac, $n=24$). Eggs size in recently deposited clutches varies from 4.2 to 6.5 mm (5.02 ± 0.55 mm, $n=37$, from five clutches) at the stages of a zygote and two blastomeres. Larvae normally hatch from the egg sac at stages 52–53 [46–48], but some of them stay inside until stages 54–55 [50–52]. Hatching larvae possess densely pigmented eyes, open mouth, short ramified gills, flattened terminal portion of fore limbs, and conical buds of hind limbs, weakly pigmented dorsal skin and gut swollen with yolk mass. The duration of the larval period from hatching till the completion of metamorphosis (stage 72 [68]) varies from 48–55 days at temperature 14–16 °C to 100–110 days at temperature 10 °C. Early larvae develop a provisory fin-like dermal outgrowth between I and II digits on the fore- and hind limbs and do not develop balancers. Advanced larvae still display a large amount of the yolk in their gut (Fig. 1B). Newly metamorphosed individuals have protruding eyes and densely pigmented skin inclusive the belly area; they retain a darkly pigmented spot at the site of the resorbed gills and the rudimental fin on the posterior portion of the tail, as well as some yolk in their digestive tract. All the larvae observed completed metamorphosis without feeding.

Skeletal development. The sequence and timing by developmental stages of the appearance of ossifications in

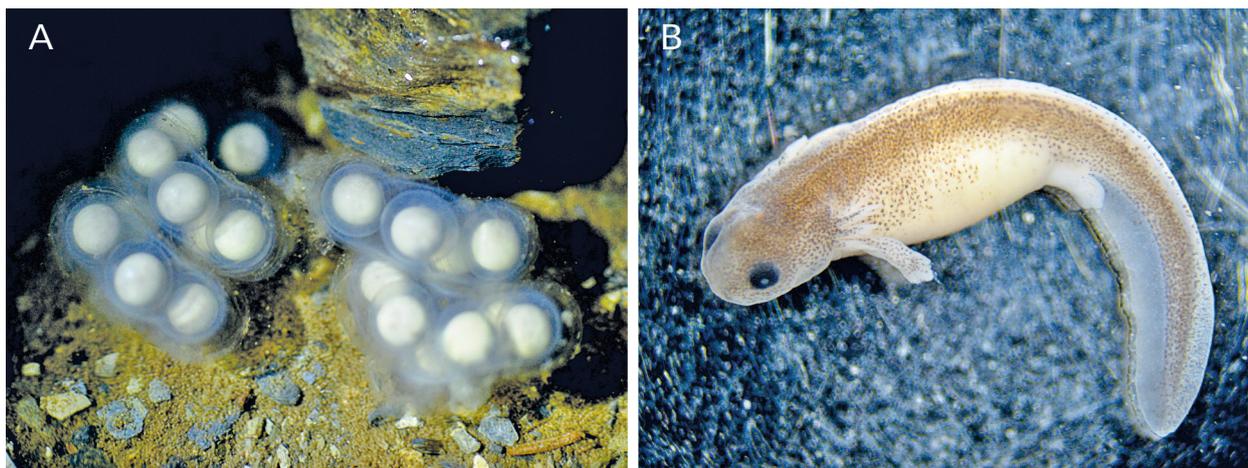


Fig. 1. A newly deposited clutch (photo by Karen S. Sarkisian) (A) and the external appearance of a two-weeks-old larva of *Hynobius formosanus* (developmental stage 58 [54–55], TL=17.9 mm) (B).

the skull and postcranial skeleton during the ontogeny of *H. formosanus* are summarized in Table 2.

By the end of embryonic development, at stages 52 [46], the skeleton of embryos or early hatching larvae consists only of cartilaginous elements which include the well-differentiated palatoquadrate and Meckelian cartilages, otic capsules, hyobranchial arches, vertebrae and well-formed cartilaginous primordia of humeral bone, scapulocoracoid, ulna and radius in the forelimb.

At stages 53–56 [48–53] embracing the hatching period and the first ten days of larval life, a set of dermal cranial ossifications makes appearance in the upper and lower jaws, skull roof, skull floor and the palate region and suspensorium (Fig. 2). In one larva (early stage 53 [47–48]) two ossification centers are clearly seen at the site of the frontal on both sides of the skull; in larvae of slightly more advanced stages (53–54 [48–50]) frontal is represented by a single elongate ossification. Teeth always precede the formation of the ossification centers of the corresponding bones. Two or three tiny tooth buds are distinguishable along the medial parts of the paired Meckelian cartilage prior to the appearance of the paired anlagen of dentary. Three tooth buds precede the ossification of the facial process of the paired premaxilla in the upper jaw. Paired vomer starts to ossify under the base of two tooth buds in the palatal region. At late stage 56 [53], about two weeks after hatching, each dentary bears three to four functional teeth (fused to the bone by their base) and a series of newly formed tooth buds lying medial to the bone. Four to five functional teeth and a new bud series are seen on each premaxilla. Each vomer bears one functional tooth and two tooth buds belonging to the next series forming medially. All teeth and germs at these stages have monocuspid crowns. No tooth buds or ossification centers are seen at the sites of the palatine or coronoid at this stage. The hyobranchial apparatus is completely cartilaginous (Fig. 2AB, 6A). In the postcranial skeleton, the ossification process affects trunk vertebrae and the forelimb: a narrow ossified zone is seen

in the middle part of the humerus. In the hind limb, cartilaginous primordia of femur, fibula and tibia are well differentiated.

Stages 57–58 [54–55] are characterized by the further growth of the earlier formed ossifications and the increase of teeth number on all dentigerous bones. On the dentary and premaxilla, a single functional tooth row becomes denser due to the maturation of teeth from the second successive series which ankylose to the bone between the first functional teeth in an alternate mode. On the vomer, two teeth belonging to the second series start to fuse to the underlying bone medial to the older functional tooth. All teeth and germs at these stages are monocuspid.

Advanced larval period (stages 60–68 [57–63]), about 20–50 days after hatching, is marked by the appearance of more ossifications in the skull (Fig. 3), including the first endochondral bones: the paired exoccipital in the occipital region followed by the mentomandibular forming in continuity with dentary bone in the symphyseal area. Walls of the cartilaginous nasal capsule are well differentiated by this time and the paired nasal forms on its top rostral to the frontal. The development of this bone begins with the formation of a small ossification center lateral to the growing rostral end of the frontal and the dorsal end of a facial process of the premaxilla (Fig. 4A). This center grows quickly expanding through the roof of the nasal capsule between frontal and premaxilla in both lateral and medial direction. Maxilla is the only dentigerous bone not preceded by the appearance of tooth germs, it remains toothless at these stages. In the lower jaw, at stages 60–61 [57–58], a single tooth bud appears medial to the dentary at the site of the future coronoid. The ossification center of coronoid forms slightly later: in larvae at the late stage 63 [58] a tiny bony plate underlying the base of the growing monocuspid tooth is seen in one or both sides of the jaw. One small monocuspid tooth germ may form at this stage medial to a functional tooth. In the larva at stage

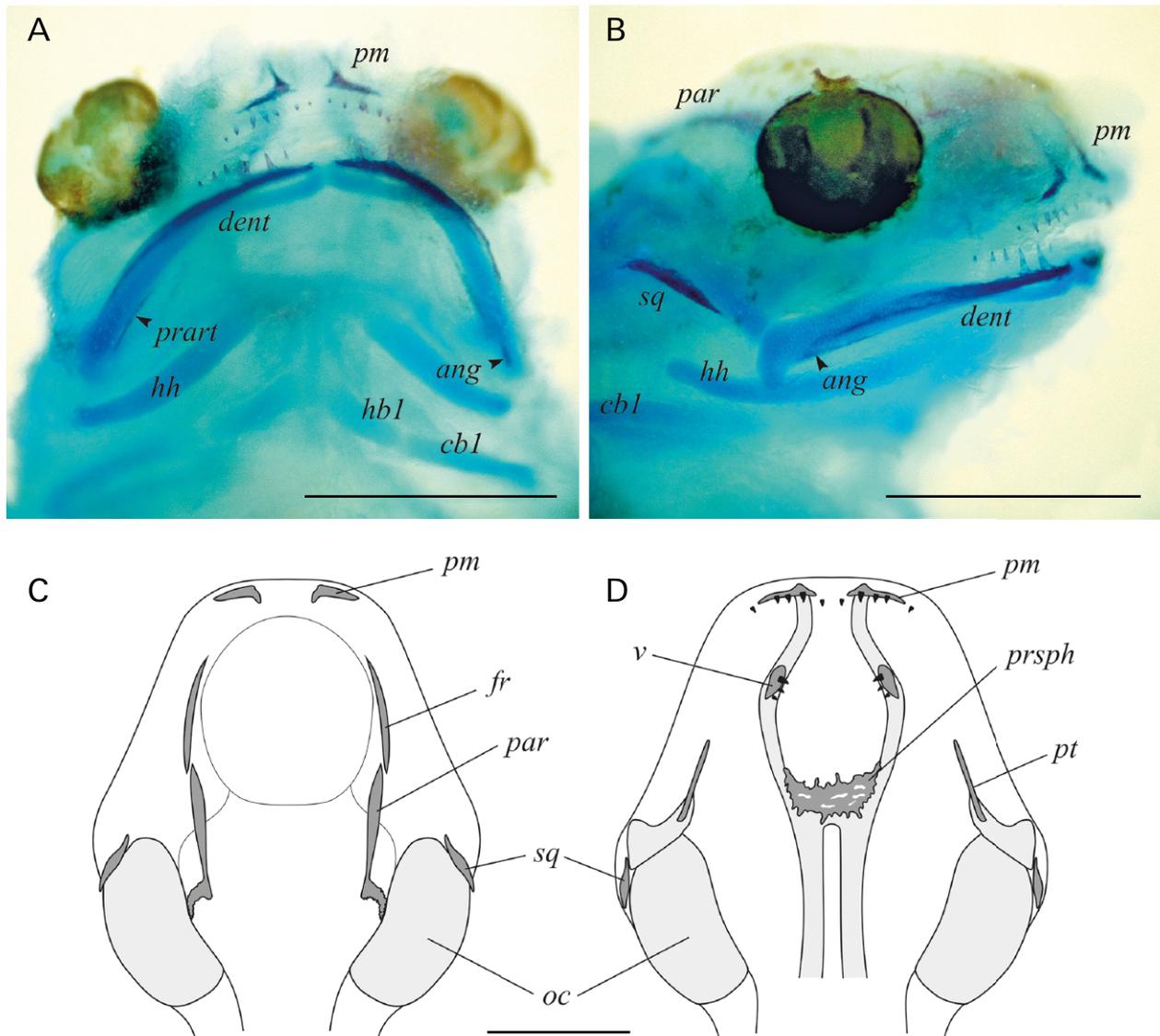


Fig. 2. Skull of the recently hatched larva of *Hynobius formosanus* (developmental stage 55[52]). **A–B:** Photograph of the entire double-stained head. **A:** Ventral view. **B:** Lateral view. **C–D:** Schematic drawing of the skull elements present in early larva, **C:** dorsal view; **D:** ventral view. Hereinafter: dark gray filling indicates bones, light gray filling indicates cartilage. Designations: *ang* – angular; *cb* – ceratobranchial; *dent* – dentary; *fr* – frontal; *hh* – hypohyal; *hb* – hypobranchial; *oc* – otic capsule; *par* – parietal; *pm* – premaxilla; *prart* – prearticular; *prsph* – parasphenoid; *pt* – pterygoid; *sq* – squamosal; *v* – vomer. Scale bar = 1 mm.

69–70 [64], coronoid was recorded in an asymmetrical condition: two fused teeth without a bony basement on one side of the jaw and a toothless tiny bone with signs of resorption on the other side (Fig. 4B). All older larvae completely lack coronoid and its dentition. Development of the dentition on the premaxilla, dentary, and vomer proceeds by the elongation of the tooth row caudally and the formation of two successive series of tooth germs lying medial to the functional tooth row. Vomerine dentition displays a polystichous arrangement of two or three functional rows (the oldest and most lateral row contains only one tooth) and one or two medial series of tooth buds. All teeth and germs at these stages are monocuspid. No tooth germs or ossification are seen at the site of a palatine (Fig. 4C). The development of the postcranial

skeleton progresses by further ossification of the vertebral column reaching the proximal caudal vertebrae and the ossification of some endochondral bones of the limbs and limb girdles (Table 2).

Stages 70–72[64–68] are characterized by the metamorphic transformation of the skull which acquires progressively its definitive shape and is complemented by several dermal and endochondral ossifications (Fig. 4D, 5, Table 2). In all larvae of stages 71–72[65–68], the prefrontolacrimal is represented by a single ossification with a distinct groove on its lateral portion underlying the nasolacrimal duct. The dentition develops on the rostral portion of the maxilla; it is produced by the premaxillary dental lamina which outgrows in the posterior direction. Vomer undergoes a significant remodeling. The

Table 2. Sequence of the ossification appearance in *Hynobius formosanus* (bones separated by comma appear simultaneously or the order of their appearance is not known).

Stage after IWASAWA & KERA, 1980	Skull and hyobranchial apparatus	Postcranial skeleton
53–54	dentary premaxilla squamosal frontal, parietal	no ossifications
55–56	pterygoid, parasphenoid, vomer, prearticular, angular	humerus, first trunk vertebrae
60–61	quadratojugal, maxilla, exoccipital coronoid (teeth)	femur
63–68	nasal coronoid (bone), mentomandibular	scapula radius ulna metacarpal II ilium, tibia, fibula, metatarsal II all trunk, sacral and 2–3 caudal vertebrae
70	<i>resorption of coronoid</i>	metacarpals I–III
71–72	septomaxilla, praefrontolacrimal prootic opisthotic vomerine bar hypobranchial 2, ceratobranchial 2	ischium metacarpals I–IV metatarsals I–IV all phalanges except IV
postmetamorphic juveniles	orbitosphenoid stapes	phalange IV up to 21caudal vertebrae all 15 rib pairs
adult	quadrate, articular, hyobranchial 1, ceratobranchial 1	mesopodial elements

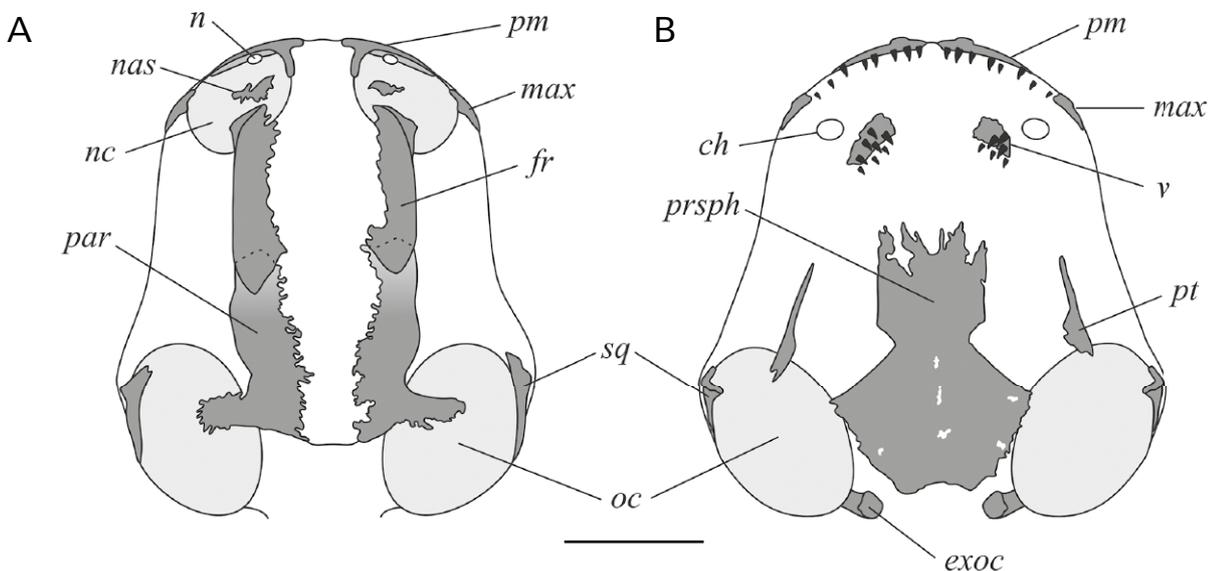


Fig. 3. Skull of the advanced larva of *Hynobius formosanus* (developmental stages 63–68 [58–63]). **A:** Dorsal view. **B:** Ventral view. Designations: *ch* – choana; *exoc* – exoccipital; *max* – maxilla; *n* – nostril; *nas* – nasal; *nc* – nasal capsule; for other designations see Fig. 2. Scale bar = 1 mm.

larval portion of the bone bearing the oldest two series of larval teeth reduces progressively via resorption proceeding from its posterolateral margin. The anterior portion of the vomer develops a wide toothless outgrowth expanding toward the premaxilla and maxilla. At the same time, the posterior bony process, vomerine bar, begins to grow in the posterior direction along the medial

part of the parasphenoid (Fig. 4D, 5B). The growth of the vomerine dental lamina with tooth germs precedes the development of the vomerine bar; tiny separate ossification centers may form under the developing teeth posterior to the vomer. Later these centers fuse with the expanding posterior end of the vomerine bar forming a continuous bony outgrowth with a dental lamina lying

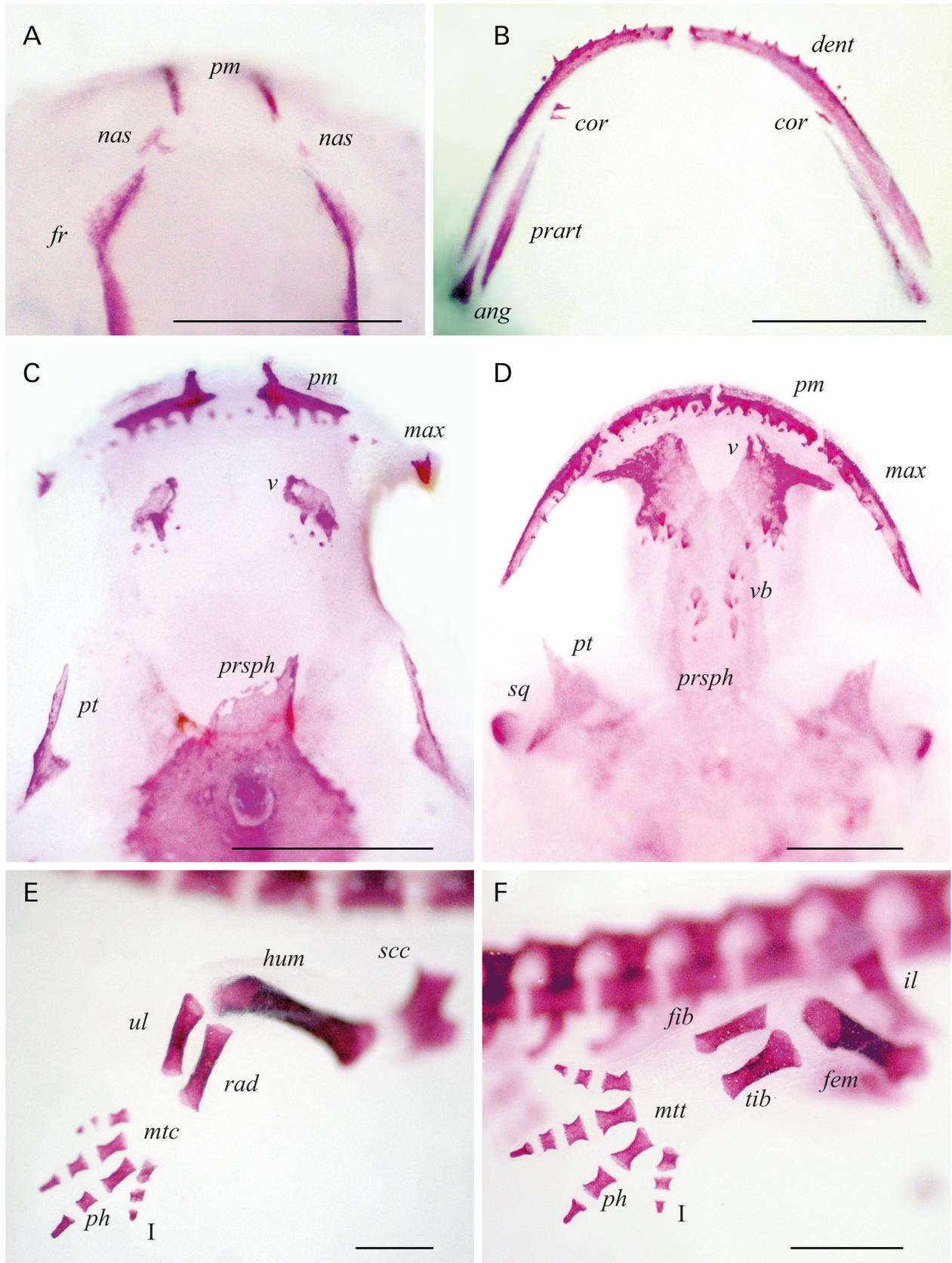


Fig. 4. Alizarin-stained skeletal elements of *Hynobius formosanus*. **A–B:** Development of nasal and coronoid in advanced larvae of *Hynobius formosanus* (stage 63[58]). **A:** Rostral part of the skull, dorsal view. **B:** Lower jaw, ventral view. **C–D:** Transformation of the vomer in the larvae during the metamorphosis, ventral view. **C:** Stage 68 [63]. **D:** Stage 71–72 [67–68]. **E–F:** The ossification state of the limb skeleton in recently metamorphosed juvenile. **E:** Forelimb. **F:** Hind limb. Designations: *cor* – coronoid; *fem* – femur; *fib* – fibula; *il* – ilium; *mtc* – metacarpals; *mtt* – metatarsals; *ph* – phalanges; *rad* – radius; *scc* – scapulocoracoid; *tib* – tibia; *ul* – ulna; *vb* – developing vomerine bar; for other designations see Fig. 2–3. Scale bar = 1 mm.

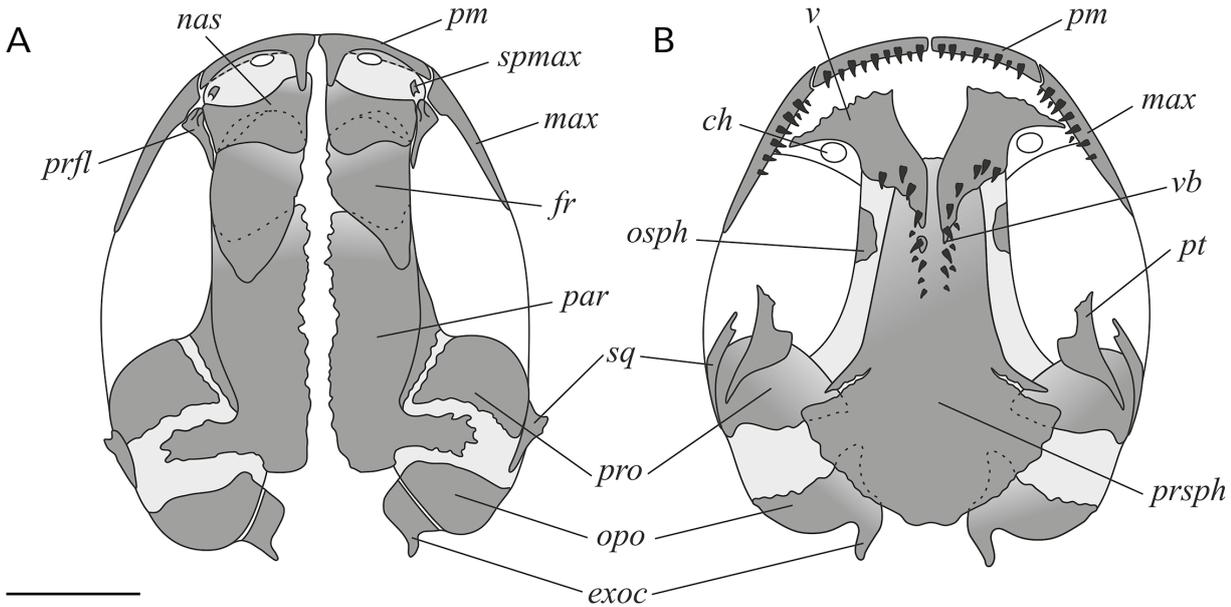


Fig. 5. Skull of the postmetamorphic juvenile of *Hynobius formosanus*. **A:** Dorsal view. **B:** Ventral view. Designations: *oph* – orbitosphenoid; *prfl* – prefrontolacrimal; *pro* – prootic; *spmax* – septomaxilla; for other designations see Fig. 2–4. Scale bar = 1 mm.

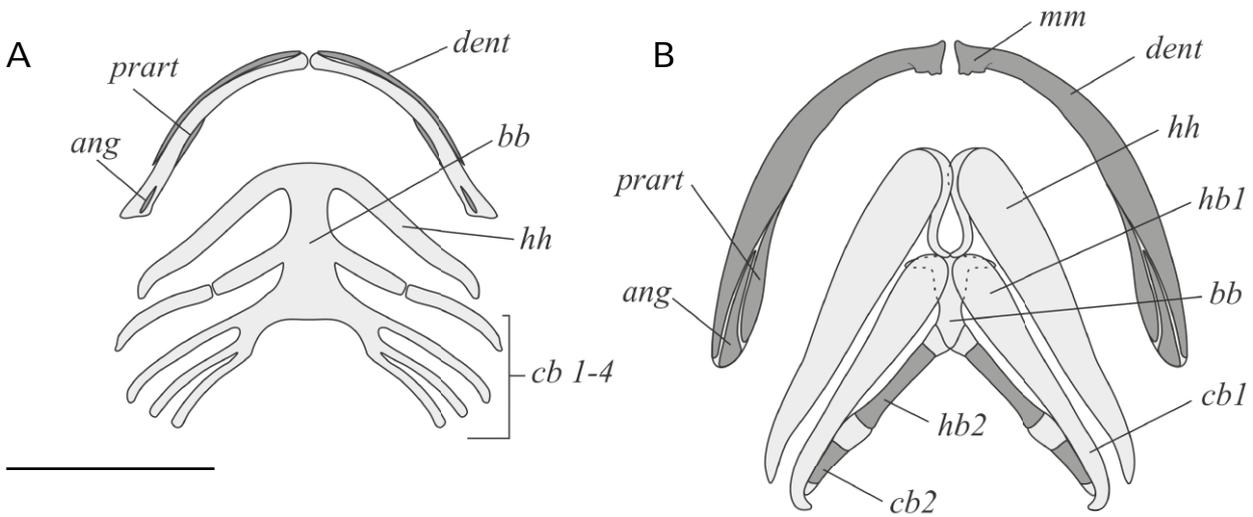


Fig. 6. Lower jaw and hyobranchial apparatus of *Hynobius formosanus*, ventral view. **A:** Larva at stage 55[52]. **B:** Postmetamorphic juvenile about one year old. Designations: *bb* – basibranchial; *mm* – mentomandibular; for other designations see Fig. 2. Scale bar = 1 mm.

lateral to it. All functional teeth at this period, including the first two to three functional maxillary teeth, and one or two successive series of tooth germs lying medial to premaxilla, dentary and vomer, are moncuspid. By the end of metamorphosis, the most young germ series seen in the dental laminae of premaxilla, maxilla, dentary and the vomerine bar, display bicuspid crowns.

The metamorphosis is accompanied also by the transformation and partial ossification of the larval hyobranchial apparatus (Fig. 6B) and the progressive ossification of limb skeleton (Table 2).

The appearance of some endochondral cranial bones, as well as the further ossification of the vertebral column, ribs and limb skeleton (except the mesopodial elements, Fig. 4EF) occurs in juvenile salamanders, from several weeks to almost one year after the completion of metamorphosis. During the early postmetamorphic life, the dentition on jaws and vomer is represented by a mixture of moncuspid and bicuspid teeth alternating in a single tooth row on the each dentigerous bone; the new series of tooth germs are distinctly bicuspid. Adult skull (Fig. 7) is characterized by an extensive roof bones

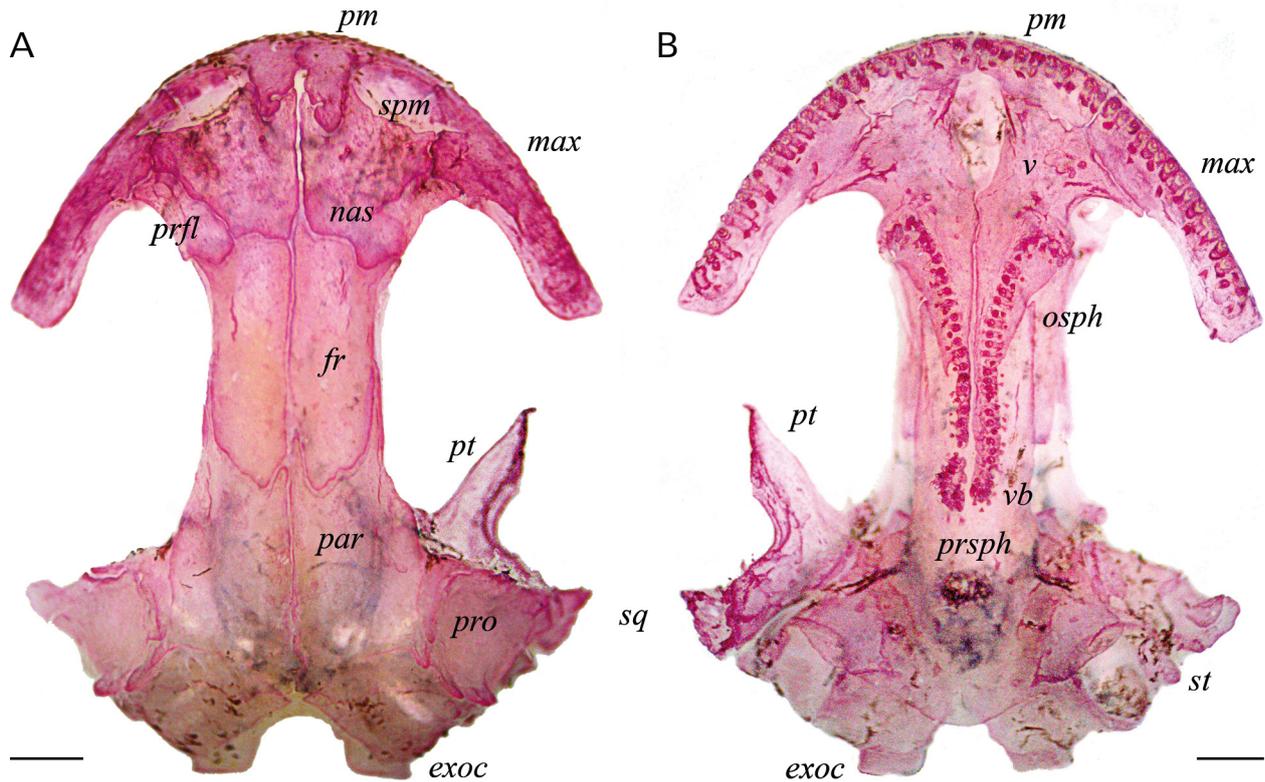


Fig. 7. Stained skull of an adult *Hynobius formosanus*. **A:** Dorsal view. **B:** Ventral view. Certain bones are removed on the left side of the skull. Designations: *st* – stapes; for other designations see Fig. 1–4. Scale bar = 1 mm.

overlapping, ample ossification of the otic region with the development of a superficial sculpture with robust spike-like outgrowths along the occipital region, and presence of some more endochondral ossifications reinforcing the jaw articulation (Table 2). The posterior end of the toothed vomerine bar running along the midline of the parasphenoid reaches the level of the anterior margin of the otic capsule (Fig 7B). The dentitions on both jaws and vomer are represented by a single functional row of bicuspid pedicellate teeth. The adult hyobranchium shows the occurrence of ossification in the first branchial arch, and appendicular skeleton develops the complete set of mesopodial bones in fore and hind limbs (Table 2).

Early skeletal development in *H. lichenatus* and *H. nigrescens*. The sequence and timing by developmental stages of the appearance of the first ossifications in the skull during the early larval ontogeny of these species are summarized in Table 3.

Larvae of both species hatch from the egg sacs with mouth which is still not open, small buds of forelimbs and the gut filled with small quantity of yolk. Hatching larvae do not display any signs of ossification in the skull or postcranial skeleton.

The first stages of skeletal development are very similar in both species. Prior to mouth opening, at stages 40–41, few tooth germs appear along the Meckelian cartilage slightly before the formation of a dentary bone.

Table 3. Sequence of the appearance of the early cranial ossifications in *Hynobius lichenatus* and *Hynobius nigrescens* (bones separated by comma appear simultaneously or the order of their appearance is not known).

Stage after IWASAWA & YAMASHITA, 1991	<i>H. lichenatus</i>	<i>H. nigrescens</i>
42–43	dentary, coronoid, vomer, palatine	dentary, coronoid, vomer, palatine
46	premaxilla, pterygoid	premaxilla, pterygoid
47	squamosal, parasphenoid, frontal, parietal, prearticular	(no data)

After the mouth opening, at stages 42–43, the appearance of other dentigerous bones is also preceded by the formation of monocuspid tooth germs in the corresponding dental laminae. The first functional teeth on the coronoid, vomer, palatine and then dentary and premaxilla form at stages 46–47, at the beginning of exogenous feeding.

The ossification of first 8–10 trunk vertebrae were recorded in the larvae of *H. lichenatus* at stage 47. No signs of ossification are seen in other postcranial skeletal elements at this stage.

Discussion

Osteology of hynobiid salamanders is of great interest for the comparative morphology because this group represents one of the most basal lineages of recent caudate amphibians (ROELANTS *et al.*, 2007; ZHANG & WAKE, 2009; PYRON & WEINS, 2011). The development of the skeleton and especially of the skull including dentition features is relatively well studied in some species, namely in *Ranodon sibiricus* (LEBEDKINA, 1979; 2004; VASSILIEVA & SMIRNOV, 2001; JÖMANN *et al.*, 2005; GREVEN *et al.*, 2006), *Salamandrella keyserlingii* (LEBEDKINA, 1979; 2004; SMIRNOV *et al.*, 2011), *Onychodactylus fischeri* (LEBEDKINA, 1981; SMIRNOV & VASSILIEVA, 2002) and *O. japonicus* (SUZUKI, 1933; OKUTOMI, 1936; VASSILIEVA *et al.*, 2013). Some fragmentary data on the skull, dentition, and hyobranchial apparatus development are available for salamanders of the genus *Hynobius*: *H. kimurae* (MISAWA & MATSUI, 1997b), *H. nebulosus* (FOX, 1959; GREVEN & CLEMEN, 1985), and *H. leechii* (Chung, 1932). Hynobiids were shown to retain numerous primitive characters in their cranial morphology and development (LEBEDKINA, 1979; 2004) and thus are considered as an important model group used in the reconstruction of urodelan phylogeny and evolution (e.g., LARSON & DIMMICK, 1993; SCHOCH & CARROLL, 2003; SMIRNOV, 2006; 2008).

Several studies discuss the phylogenetic position of the Taiwanese *Hynobius* species within the phylogeny of this genus; they all agree that these species form a monophyletic clade, distantly related to Japanese lotic *Hynobius* species, as well as to the most basal lentic *Hynobius* (*Satobius*) *retardatus*, sister clade to all other *Hynobius* (ZHANG *et al.*, 2006; ZHENG *et al.*, 2011; LI *et al.*, 2011; WEISROCK, 2012; ZHENG *et al.*, 2012). Such phylogenetic position might indicate that the Taiwanese *Hynobius* species represent a clearly distinct evolutionary lineage, adapted to conditions of extreme highlands of Taiwan, at the southernmost border of hynobiid distribution.

The results obtained in the present study show that *H. formosanus* differs from all other studied hynobiids by several important features of its skeletal ontogeny. The most striking peculiarity is the absence of the palatine bone at any developmental stages, whereas this dentigerous bone is present as a portion of a palatopterygoid in all hynobiid larvae studied to date (ROSE, 2003). The greatly reduced state of the coronoid is also noteworthy: this bone occurs in *H. formosanus* larvae only as a very small rudimental ossification with an underdeveloped dentition. Moreover, the observed heterochronic alteration of the sequence of cranial bones development in comparison with other hynobiids is noteworthy. Finally, the absence of the separate lacrimal, recorded in *H. formosanus*, is also unusual for hynobiids. We presume that all these features are closely associated with specific traits of life history in this species discussed below.

First, the development of *H. formosanus* displays a certain degree of embryonization in comparison with other *Hynobius* species, in particular the lentic-breeding *H. lichenatus* and *H. nigrescens*. Namely, *H. formosanus* possesses much larger eggs (from 4.25 ± 0.06 mm (KAKEGAWA *et al.*, 1989) to 5.02 ± 0.55 mm (our data) in diameter) than *H. lichenatus* (2.8 ± 0.2 to 3.2 ± 0.3 mm, TAKAHASHI & IWASAWA, 1990) and *H. nigrescens* (2.17 to 2.92 mm, TAKAHASHI & IWASAWA, 1988). Also, *H. formosanus* has a rather prolonged embryonic development which takes about three months (KAKEGAWA *et al.*, 1989), whereas in lentic *H. nigrescens* it takes around 10–20 days depending on temperature (IWASAWA & YAMASHITA, 1991). Larvae of *H. formosanus* leave the egg sac at stages 46–52 I&Y, whereas *H. nigrescens* larvae hatch at much earlier stages 40–41 (IWASAWA & YAMASHITA, 1991). To date, *H. formosanus* remains the sole member of the Hynobiidae which may complete metamorphosis relying only on endogenous feeding during the whole larval period (KAKEGAWA *et al.*, 1989; our data). Most other studied hynobiids start the exogenous feeding soon after hatching; the few exceptions known are the lotic-breeding *H. kimurae* and *Onychodactylus* spp.: their larvae also hatch with abundant yolk reserves in their gut and begin to feed only at very advanced developmental stages (REGEL' & EPSHTEIN, 1975; IWASAWA & KERA, 1980; AKITA, 2001).

It is remarkable that both *Onychodactylus* species studied and *H. kimurae* also display certain reduction of transient skull elements. In *O. fischeri* and *O. japonicus*, coronoid is always rudimentary and sometimes edentate; the palatine portion of palatopterygoid is narrowed and bears reduced dentition (SMIRNOV & VASSILIEVA, 2002; VASSILIEVA *et al.*, 2013), whereas in most hynobiids it is represented by widened plate bearing polystichous tooth-field, for example, in *R. sibiricus* and *S. keyserlingii* (LEBEDKINA, 1979; 2004; VASSILIEVA & SMIRNOV, 2001) and *H. nebulosus* (GREVEN & CLEMEN, 1985). In *H. kimurae*, a palatine is reported to be fully developed, but the coronoid is described as “vestigial” (MISAWA & MATSUI, 1997b).

At the same time, more or less pronounced reduction of provisory skull bones is observed in other salamanders with embryonized development, for example, in *Rhyacotriton olympicus* (WORTHINGTON & WAKE, 1971) and *Amphiuma* (HAY, 1890). Among plethodontids, the direct-developers hatching at the stage of metamorphosis do not develop pterygopalatine and coronoid at any stage of their development; whereas partially embryonized species hatching at advanced larval stages and pursuing endogenous feeding also lack the coronoid and develop only the pterygoid portion of the palatopterygoid, which disappears at metamorphosis (MARKS, 2000). Thus, the observed reductions of the larval ossifications in the skull of *H. formosanus* may be regarded as the consequence of the advanced embryonization of their ontogeny. Dentigerous palatines and coronoids are believed to play an important role in the feeding of urodelan aquatic larvae (LEBEDKINA, 1964; DEBAN & WAKE, 2000). Likely,

these elements lose their functional importance in direct-developing salamander or in species with non-feeding larvae and may gradually reduce on the evolutionary pathway leading to the progressive embryonization or endogenous larval feeding.

Also, *H. formosanus* differs from most studied hynobiids by an altered sequence of bony skull formation. Thus, coronoid, vomer and palatine are among the very first bones appearing in *R. sibiricus* and *S. keyserlingii* (LEBEDKINA, 1979; 2004; VASSILIEVA & SMIRNOV, 2001; SMIRNOV *et al.*, 2011) as well as in *H. lichenatus* and *H. nigrescens* (this study), and always form before the premaxilla and squamosal. In *H. formosanus*, lacking the palatine, the development of vomer and especially of coronoid is delayed and occurs after the premaxilla and squamosal are already present. Interestingly, the same ossification sequence plus delayed palatine formation are observed in the skull of *O. japonicus* (VASSILIEVA *et al.*, 2013). Moreover, similar heterochronies affecting the early stages of cranial development are revealed in the representatives of another urodelan lineage, true salamanders (Salamandridae) *C. lusitanica* and *M. caucasica*: in these partially embryonized species the development of the coronoid, vomer, and palatine is retarded compared to non-embryonized salamandrids (such as various newts) (VASSILIEVA *et al.*, 2011; VASSILIEVA & SERBINOVA, 2013). Coronoid and palatine are provisory elements in larvae of urodelans which complete metamorphosis; vomer is preserved in adult salamanders but undergoes a considerable transformation during metamorphosis, including the resorption of larval bony tissues (LEBEDKINA, 1979; 2004; ROSE & REISS, 1993). We presume that the delayed development of toothed provisory bones in salamanders with partially embryonized ontogeny may be caused by the diminishing of their functional importance at early larval stages. At the same time, the development of bones forming the jaws (dentary, premaxilla) and jaw suspension (squamosal) in embryonized salamanders is less delayed in comparison with provisory bones, possibly, because of the need to reinforce the jaw apparatus and the whole skull with well-developed ossifications by the start of exogenous feeding.

The late stages of skull ontogeny in *H. formosanus* also display certain heterochronies in comparison with other hynobiids. For example, the late-forming endochondral bones like quadrate, orbitosphenoid, and stapes normally appear before or during the metamorphosis in *R. sibiricus*, *S. keyserlingii* (LEBEDKINA, 1979; 2004; SMIRNOV & VASSILIEVA, 2002), and *O. fischeri* (SMIRNOV & VASSILIEVA, 2002), whereas in *H. formosanus* they form only after metamorphosis is fully completed. Presumably, such shift of the latest events of larval cranial ontogeny into the postmetamorphic period may be explained by relatively short larval period in *H. formosanus*, especially in comparison with other lotic-breeding hynobiids. Thus, the development from hatching to the completion of metamorphosis under laboratory conditions takes more than 200 days in *R. sibiricus* (LEBEDKINA, 1964) and several months in *O. japonicus* (IWASAWA & KERA, 1980). In rela-

tively fast-developing lentic species, larval period takes about 2.5–3 months under room temperature in *S. keyserlingii* (observations of A. Vassilieva) and *H. nigrescens* (IWASAWA & YAMASHITA, 1991). In *H. formosanus*, metamorphosis is completed in 48–55 days after hatching under similar temperature conditions. We presume that temporal limitations associated with an exclusively endogenous feeding and exhaustion of yolk reserves entail the relative abbreviation of larval period and acceleration of metamorphosis in this species, with a consequent transition of certain late-larval or metamorphic events to the postmetamorphic developmental phase.

Certain acceleration of larval skull development might also explain the unusual development of prefrontolacrimal, a dermal bone reinforcing the cranial roof in *H. formosanus*. The separate ossification center of lacrimal was not found in *H. formosanus*, although the morphology of the bone occupying the place of the prefrontal in this species indicates its complex nature: the occurrence of a groove underlying the nasolacrimal duct on the distal end of the bone evidences its homology with lacrimal, since the development of lacrimal was shown to be strongly connected with the nasolacrimal duct (MEDVEDEVA, 1959; 1960). The separate lacrimal is characteristic for all hynobiids studied to date, including *R. sibiricus*, *S. keyserlingii* (LEBEDKINA, 1979; 2004), *Pachyhynobius shangchengensis* (CLEMEN & GREVEN, 2009), *Onychodactylus* spp. (SMIRNOV & VASSILIEVA, 2002; VASSILIEVA *et al.*, 2013), and several *Hynobius* species (NISHIO *et al.*, 1987). Previously it was shown that the experimental acceleration of metamorphosis in *S. keyserlingii* by hormonal treatment may result in the reduction of number of ossification centers in the developing skull bones, apparently because of the precocious fusion of bone primordia before the start of their mineralization (SMIRNOV *et al.*, 2008). Assumingly, the shortening of larval development in *H. formosanus* leads analogously to the accelerated fusion of the primordia of the prefrontal and lacrimal with the formation of a complex bone. Interestingly, the complex origin of the prefrontal was proved for salamandrids, and the development of a single bone from several ossification centers homologous to prefrontal and lacrimal bones is admittedly explained by similar heterochronic mechanisms (VATER, 2007).

As a conclusion, we consider the observed reductions and heterochronies in the cranial development in *H. formosanus* to be a result of the ontogenetic repatterning associated with the evolution of life history in this species toward the embryonization and abbreviation of larval period. To date, *H. formosanus* possesses the most deviated ontogeny among all the hynobiids studied, although we may expect similar developmental peculiarities in other Taiwanese species, such as *H. sonani*, which apparently differs by a rather advanced degree of embryonization and abundant yolk reserves at hatching (KAKEGAWA *et al.*, 1989). The unique ontogeny of *H. formosanus* may be associated with the rather specific breeding biology of this species, preliminarily described by CHANG *et al.*, 2009 and deserving a special extended study.

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