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Ontogeny of skeletal ossification in *Labeo calbasu* (Hamilton, 1822) using differential fluorescence staining technique

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Abstract

The present study was carried out to study the larval skeletal development in *Labeo calbasu* by using a modified double skeletal staining technique with Alizarin red and Alcian blue. The larval samples were obtained after induced breeding of wild *L. calbasu* germplasm from the River Ganga. Samples from 2 to 20 dph (day post hatching) were preserved in 4% neutral phosphate buffered formalin solution. Alizarin red and Alcian blue were used to stain the bony and cartilage parts of the skeleton, respectively. The size of the specimens ranged from 6.6 ± 0.16 to 15.6 ± 1.15 mm. The development of skeleton was observed at very early stages. A straight notochord throughout its length and origin of caudal fin rays were seen on 2 dph. The ventral spines, unbranched caudal fin rays and hypurals at ventral side of notochord were clearly visible from 4 dph. Most of the head skeletal elements and vertebral column with vertebral centrum and neural spines started appearing at 4 dph. The dorsal and caudal fins with branched rays and the opercular and jaw bones started ossifying between 10 and 20 dph. The present study gives an idea about the skeletal development process as well as detects the skeletal abnormalities in Indian major carp, *L. calbasu*.

KEYWORDS
Labeo calbasu, ontogeny, ossification, skeletal system, staining

1 | INTRODUCTION

Fish skeletal system is composed of exoskeleton and endoskeleton, where exoskeleton comprises scales and fin rays. The un-segmented spines and segmented branched soft rays are the parts of the fin rays. The endoskeleton is the completely ossified part of the skeletal system that forms the framework of the body. It consists of the axial skeleton and the appendicular skeleton. The axial skeleton includes skull, vertebral column and ribs, and the appendicular skeleton includes the girdle and the supporting elements of fins. The multiplicity of the bones with their complex arrangement in fish requires a vivid study on their ontogeny for the characterization of the skeletal system as well as the differential identification of species based on the skeletal arrangement (Imran *et al*., 2014). In teleosts, sequence of functional changes in physiological behaviour such as feeding, respiration and swimming is accompanied with parallel sequence of morphological changes in both larvae and juveniles (Yu & Kim, 2016). Therefore, a detailed knowledge about the developmental osteology is important for phylogenetic inferences about the functional relationships among teleost taxa and the environmental preferences of different developmental stages of fish (Koumoundouros *et al*., 1997, 1999). The skeletal abnormalities originating at the early larval and juvenile stages are mainly due to the unfavourable abiotic conditions which affect the morphology and growth of fishes. Therefore the ontogenic study of the skeletal system will not only help in taxonomy but also in the early detection of any skeletal defects in the larvae during the artificial seed production. This will help in finding the possible causes and the mitigation strategies of the skeletal abnormalities to promote effective aquaculture and resources management (Koumoundouros *et al*., 1997). Differential fluorescent staining technique is one of the methods to study the development of bones and cartilages, track the

TABLE 1 Developmental sequence of ossification (Balon, 1985) in *Labeo calbasu*

		Total length (mm)						
		Preflexion larvae		Flexion larvae	Postflexion larvae			Juvenile
		Mean TL + S.E., $n = 5$						
Skeletal elements identified		6.6 ± 0.16 (2 dph)	7.08 ± 0.25 (4 dph)	8.47 ± 0.28 (6 dph)	9.32 ± 0.41 (7 dph)	11.52 ± 0.68 (11 dph)	13.73 ± 1.26 (15 dph)	15.6 ± 1.15 (20 dph)
Cranium	Hyomandibular							
	Opercle							
	Lateral ethmoid							
	Frontal							
	Parasphenoid							
	Sphenotic							
	Pterotic							
	Palatine							
	Orbit							
	Suborbital							
	Brancheostegal							
	Scalpula							
	Clavicle							
	Pectoral girdle bone							
	Pectoral fin rays							
Jaw bone	Upper jaw							
	Lower jaw							
	Maxillary							
	Premaxillary							
	Dentary							
	Articular							
	Angular							
Vertebrae	Vertebral column							
	Parapophysis							
	Neural spine							
	Hemal spine							
	Dorsal fin spine							
	Pelvic fin spine							
	Anal fin spine							
	Abdominal ribs							

TABLE 1 (Continued)

		Total length (mm)						
		Preflexion larvae Mean TL + S.E., $n = 5$		Flexion larvae	Postflexion larvae			Juvenile
Skeletal elements identified		**6.6 ± 0.16 (2 dph)**	**7.08 ± 0.25 (4 dph)**	**8.47 ± 0.28 (6 dph)**	**9.32 ± 0.41 (7 dph)**	**11.52 ± 0.68 (11 dph)**	**13.73 ± 1.26 (15 dph)**	**15.6 ± 1.15 (20 dph)**
	Interneural spine							
	Interhemal spine							
Caudal skeleton	Hypural (fused)							
	Caudal fin spine							
	Urostyle							
	Hypural(1–5)							
	Parhypural							
	Epural							
	Uroneural							
	First preural							
	Second preural							
	Rudimentary neural arch							

Note: n = 5. Values are mean ± S.E. Blue colour represents ossified state. S.E., standard error; TL, total length.

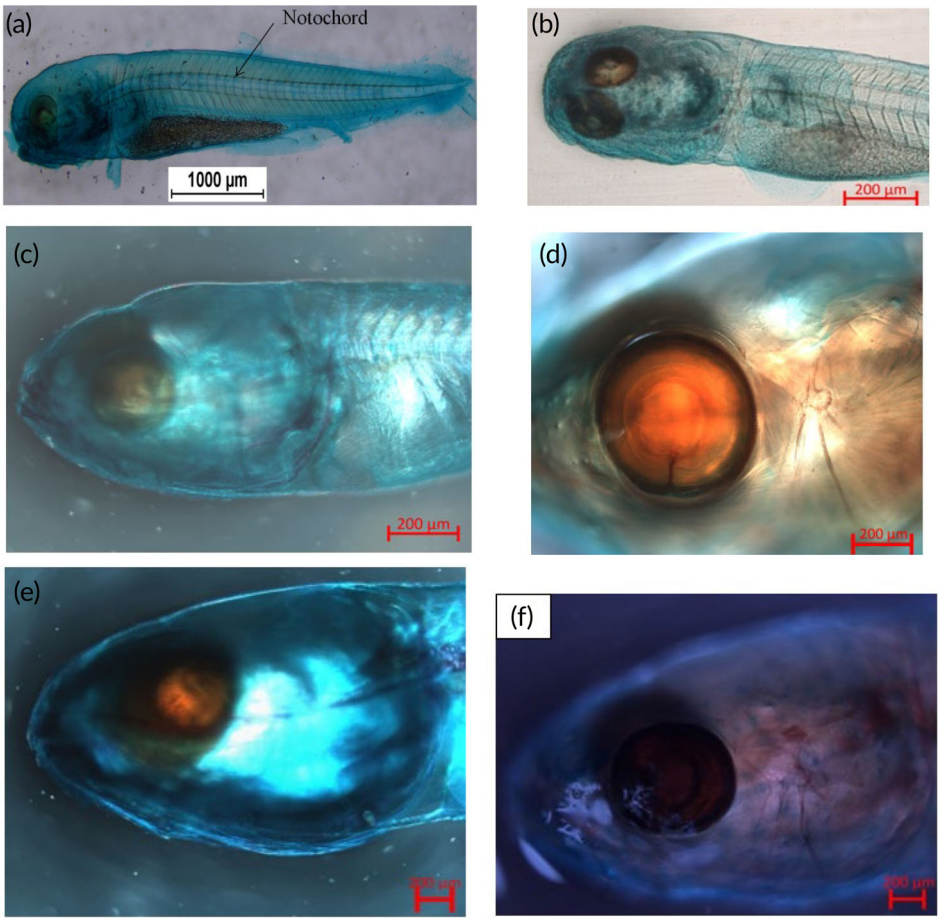

FIGURE 1 Ossification of cranium in *Labeo calbasu* under a light microscope. (a) 2 dph, 6.6 mm total length (TL) – clear notochord is seen; (b) 4 dph, 7.08 mm; (c) 7 dph, 8.47 mm; (d) 11 dph, 11.5 mm; (e)15 dph, 13.73 mm; (f) 20 dph, 15.6 mm

possible deformity and to identify closely related fish species of same family during its larvae and juvenile stage (Koumoundouros *et al*., 1997; Park *et al*., 2016; Seo *et al*., 2018). The differential fluorescent staining technique was used in this study to understand skeletal development and to detect skeletal abnormalities in Indian major carp *Labeo calbasu*, which is an important candidate species for riverine capture fisheries of the Indian sub-continent as well as from aquaculture point of view in South-east Asia (Dwivedi, 2009; Gupta & Banerjee, 2015; Imran *et al*., 2014).

2 | MATERIALS AND METHODS

2.1 | Sample collection and preparation

The examined material included preflexion larvae (2 and 4 dph), flexion larvae (6 dph), postflexion larvae (7, 11 and 15 dph) and juvenile (20 dph) of *L. calbasu* obtained through induced breeding of wild fish collected from the River Ganga (23° 07′ 31.1″ N 88° 28′ 20.9″ E). Brood fish were kept in 10,000 l cement tanks for 2 days prior to induced breeding. After hatching, larvae were placed in fibreglass reinforced plastic rearing tanks and were fed with mixed zooplankton twice daily *ad libitum*. The 10 larvae in every sampling day were collected from 2 to 20 dph from the rearing tanks and were fixed immediately in 4% neutral buffered formalin. After 5 days, the specimen were washed with running tap water and dehydrated for 60 min in each graded concentration (50%, 70%, 90% and 100%) of ethanol.

2.2 | Staining of fish larvae

The dehydrated larvae were stained according to the modified simultaneous differential staining technique with Alizarin red and Alcian blue (Aliesfehani, 2015). Briefly, the preparation of stock solution involved four steps. First 0.3% Alcian blue in 70% ethanol and 0.12% Alizarin red S in 95% ethanol were prepared. The staining solutions were filtered using 0.3 mm Whatman filter paper. The working staining solution was prepared by mixing one part of 0.3% Alcian blue, one part of 0.12% Alizarin red, 70 part of 70% ethanol and one part of glacial acetic acid (1:1:70:1). The macerating solution was prepared by adding 1 g KOH and 0.9 g NaCl in 100 ml water. The specimens were incubated at least five times volume of specimen in the mixed staining solution for 3 days for staining the bones with Alizarin red S and the cartilage with Alcian blue (Aliesfehani, 2015). Then, the stained samples were kept in the macerating solution for bleaching. After 1 day of incubation, the solution was changed and the specimens were incubated in the fresh solution for 2–3 days until the specimens became purple

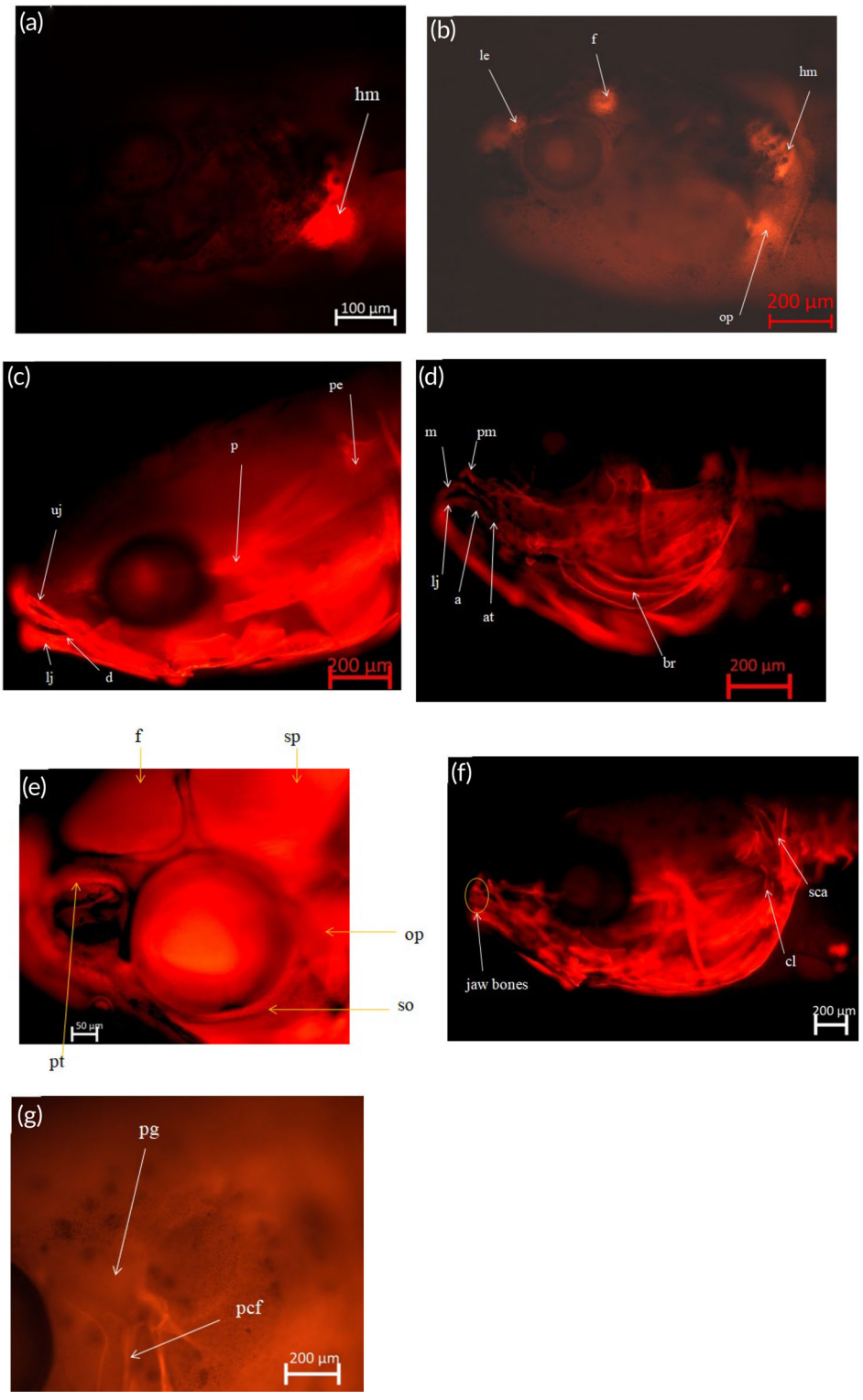

FIGURE 2 Ossification of cranium and pectoral girdle in *Labeo calbasu* under fluorescent microscopy. (a) 2 dph, 6.6 mm total length (TL); (b) 4 dph, 7.08 mm; (c) 6 dph, 8.47 mm; (d) 7 dph, 9.32 mm; (e)11 dph, 11.52 mm; (f)15 dph, 13.73 mm; (g) 20 dph, 15.6 mm; f: frontal; hm: hyomandibular; op: opercle; uj: upperjaw; lJ: lower jaw; p: parasphenoid; pe: pterotic; d: dentary; m: maxilla; pm: premaxilla; a: angular; at: articular; br: brancheostegal rays; sca: scalpula; cl: clavicle; pg.: pectoral girdle; pcf: pectoral fin

and transparent. For clearing and hardening of the tissue, the double stained specimens were kept in graded concentrations of glycerol (30%, 50%, 70%, 90% and 100%) in 1% KOH (potassium hydroxide) for 2–3 days. The specimens were preserved in 100% glycerol solution by adding phenol crystals to prevent spoilage and fungal growth.

2.3 | Microscopic observation

The stained samples were photomicrographed at a CCD basic resolution of 5 megapixels under an epifluorescent microscope (Carl Zeiss, Axioscope.A1 microscope, Oberkochen, Germany) using a set of 15 optical filters (546–590 nm) and a coupled AxiocamICc5 CCD colour camera fitted

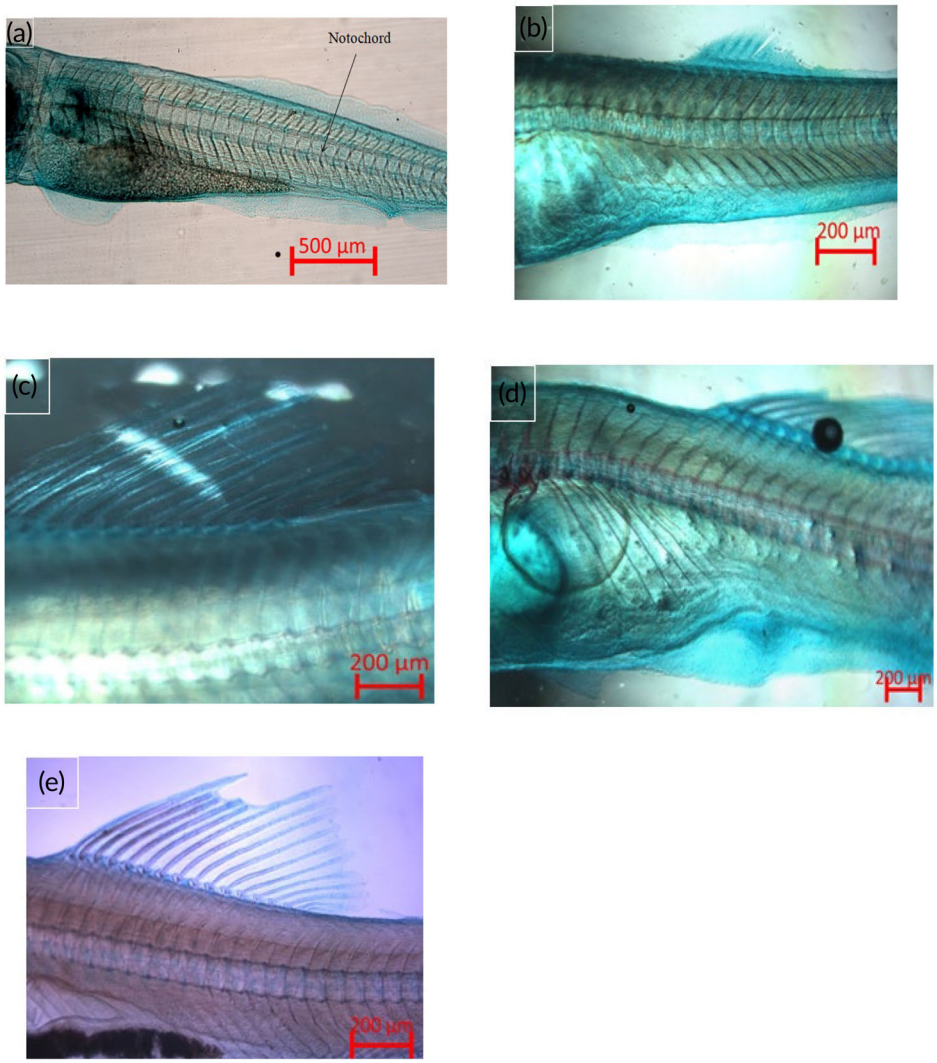

FIGURE 3 Ossification from the beginning of notochord to caudal peduncle in *Labeo calbasu* under a light microscope. (a) 2 dph, 6.6 mm; (b) 7 dph, 9.32 mm (c) 11 dph, 11.5 mm; (d) 15 dph, 13.7 mm; (e) 20 dph, 15.6 mm

with a Sony ICX 655 sensor (Tokyo, Japan) with 400–700 nm spectral sensitivity and RGB filters.

2.4 | Physio-chemical parameters of water

The physio-chemical parameters like water temperature, dissolved oxygen, pH, conductivity were measured by employing a digital meter (YSI-PRO-DSS, Ohio, USA) every week. Free CO_2, alkalinity and hardness were assessed by the titrimetric method using a standard method (APHA, 2012).

2.5 | Statistical analysis

The data obtained were subjected to descriptive analysis using MS-Excel 2003. Values presented are means ± S.E. where $n = 5$.

2.6 | Ethical statement

The present work was carried out with 2–20 old fish larvae. Fish larvae were killed after anaesthetised using standard anaesthesia (clove oil). No drug, medicine and chemicals were used in animal experiments. The rearing of fish larvae does not come under animal ethical committee approval.

3 | RESULTS

The gradual and progressive development of bones (stained red) and cartilages (stained blue) of the cranium, jaw, pectoral girdle, visceral skeleton, vertebrae, fins and caudal skeleton of *L. calbasu* larvae (6.6 ± 0.16 to 15.6 ± 1.15 mm) was observed from 2 to 20 dph under light and fluorescent microscope. The developmental observations are summarized following using standard terminology (Balon, 1985) (Table 1).

3.1 | Cranial part

The cranial part is composed of cranium and visceral skeleton. On 2 dph [6.6 ± 0.16 mm in total length (TL)], a straight notochord throughout its length is visible under light microscope (Figure 1a). The ossification of hyomandibular bone, articulation of lower jaw and operculum had started to ossify on 2 dph (Figure 2a). The opercle bone, frontal bone and lateral ethmoid began to ossify by 4 dph (7.08 ± 0.25 mm TL) (Figure 2b). The lower jaw forming the dentary, the upper jaw and the parasphenoid bone forming the base of the cranium started ossifying on 4 dph (Figure 2c). The toothed premaxillary and the toothless maxillary part of the upper jaw as well as the articular and angular bones of the lower jaw began ossification on 7 dph (9.32 ± 0.41 mm TL). Similarly, the brancheostegal rays supporting the gill membrane began ossifying as curved bones just below the operculum and behind the lower jaw by 7 dph (Figure 2d). Bones associated with the head, such as the frontal, sphenotic, pterotic, palatine, orbit, suborbital and opercular bones, started ossification by 4 dph and were completely ossified by the end of 11 dph (11.52 ± 0.68 mm TL) (Figure 2e). The clavicle and scalpula started to ossify and form the shoulder girdle by 15 dph (13.73 ± 1.26 mm TL) (Figure 2f). Subsequently, the frontal bone in the cranium and branchyostegal rays in the hyoid arches, and pectoral girdle supporting the pectoral began ossification by 20 dph (15.6 ± 1.15 mm TL) (Figure 2g).

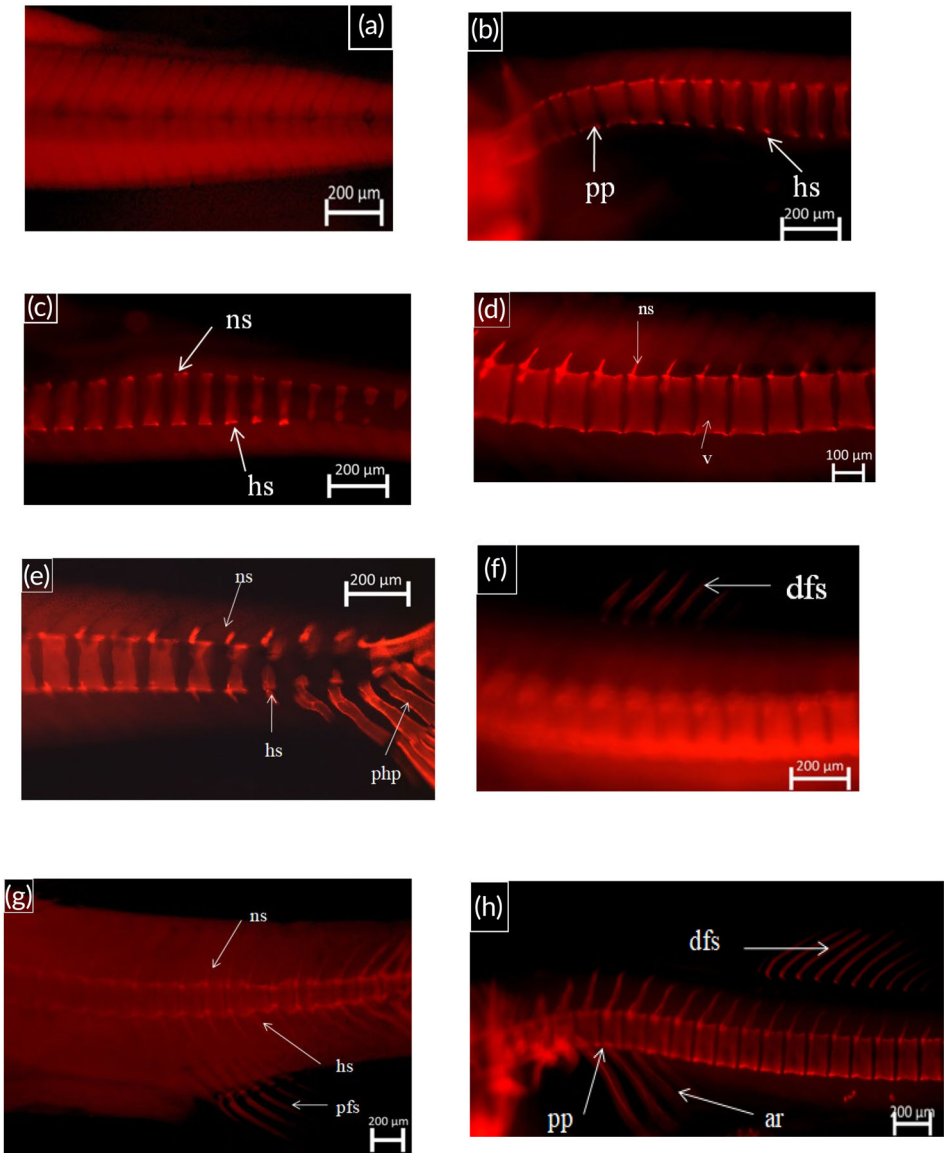

FIGURE 4 Ossification and development of the vertebrae and spines and soft fins in *Labeo calbasu* from the beginning of notochord to caudal peduncle under fluorescent microscopy. (a) 4 dph, 7.08 mm; (b) 6 dph, 8.47 mm; (c) 6 dph, 8.47 mm; (d) 7 dph, 9.32 mm; (e) 7 dph, 9.32 mm; (f) 7 dph, 9.32 mm; (g) 11 dph, 11.5 mm; (h) 11 dph, 11.5 mm; (i) 15 dph, 13.7 mm; (j) 20 dph, 15.6 mm; (k) 20 dph, 15.6 mm; ns: neural spine; v: vertebrae; hs: hemal spine; ins: interneural spines; ihs: interhemal spines; dfs: dorsal fin spines; pp.: parapophysis; ar: abdominal ribs; pfs: pelvic fin spine; afs: anal fin spines; php: parhypural; u: urostyle; rna: rudimentary neural arch; pu2: first preural; pu3: second preural

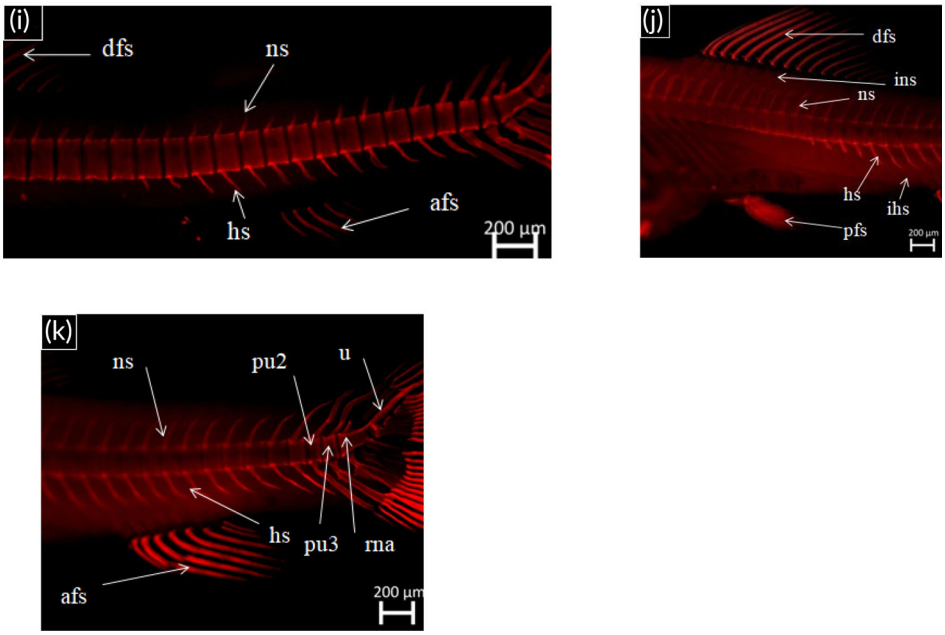

FIGURE 4 (Continued)

3.2 | Middle part

The middle part starts from the beginning of vertebral column; includes the vertebral column, dorsal fin, pectoral fin, pelvic fin and the anal fin; and ends at the caudal peduncle. The notochord was seen as a flexible rod-shaped structure spanning the length of the body of larvae on 2 dph with the total length 6.6 ± 0.16 mm and 3 dph under light microscope (Figure 3a,b). No ossified part in the middle part was observed at 2–4 dph (Figure 4a). The ossification process of vertebrae started by 6 dph when the larvae attained the total length of 8.47 ± 0.28 mm (Figure 4b). From 6 dph, the formation of vertebrae started replacing the notochord gradually from the rostral to the caudal end (Figure 4b–k). Ossification of the parapophysis, the hemal spines and the neural spines had started by 6 dph (Figure 4b,c) and proceeded gradually together with the vertebrae (Figure 4d,e). The rays of dorsal fin, pectoral fin, pelvic fin and anal fin did not start ossification until the 7 dph; nonetheless, the spines of the dorsal fins started to appear at 7 dph (Figure 4f). Between 11 and 15 dph (11.52 ± 0.68 to 13.73 ± 1.26 mm), the abdominal vertebrae ossified with the formation of a long pair of abdominal ribs (Figure 4h). The spines of the pelvic and anal fins started to ossify from 11 dph (Figure 4g,i).The interneural and interhemal spines were completely ossified by 20 dph at the total length 15.6 ± 1.15 mm (Figure 4j).

3.3 | Caudal part

The caudal skeleton includes the caudal peduncle and the caudal fin rays. The cartilaginous development of the caudal part started by 4 dph. The cartilaginous development of urostyle started by 7 dph (Figure 5d) as seen under the light microscope. Nonetheless, the absence of fluorescence from the urostyle by this time indicated the lack of bone development. The development of caudal fin started during the progression of ossification of vertebral column. The caudal complex comprised of paired uroneural (un), an unpaired parhypural (php), five unpaired hypurals (hy1–hy5) and a single epural (ep), all of which were initially cartilaginous in nature. At 4 dph (7.08 ± 0.25 mm TL), there was no ossification of the hypurals which remained fused until 6 dph. The fused hypurals and the spines of the caudal fin started ossification from 6 dph (Figure 6a). The articulated hypurals started separating into five hypural bones by 7 dph (9.32 ± 0.41 mm TL) with the parhypural positioned beneath the 5th hypural bone (Figure 6b). The ventral portion of the caudal skeleton was formed by the parhypural, to the dorsal edge of which the 5th hypural was articulated ventrally by fibrous tissues and other supporting elements (Figure 6c). The last caudal vertebra was modified into pleurostyle (urostyle) and was turned upward along with the diural; that is, the first preural (pu2) and the second preural (pu3) of the axial skeleton provided caudal support (Figure 6c). The epural (ep) is a single rod-shaped bone that lies dorsal to the urostyle and is ossified clearly between 11 and 15 dph (Figure 6c,d). By 11 dph, the caudal fin clearly differentiated into the dorsal and ventral lobes (Figure 6c). The 10 + 9 arrangement of the branched caudal lepidotrichia, which were supported by the neural and haemal spine of the diural, epural, pleurostyle, hypurals and the parhypural, was found clearly between 15 and 20 dph (Figure 6d,e).

3.4 | Physio-chemical parameters of the rearing tanks

The success of larval rearing of any fish species depends on water parameters as well as water quality management. Mean value of temperature, pH, dissolved oxygen (DO), conductivity, total alkalinity and

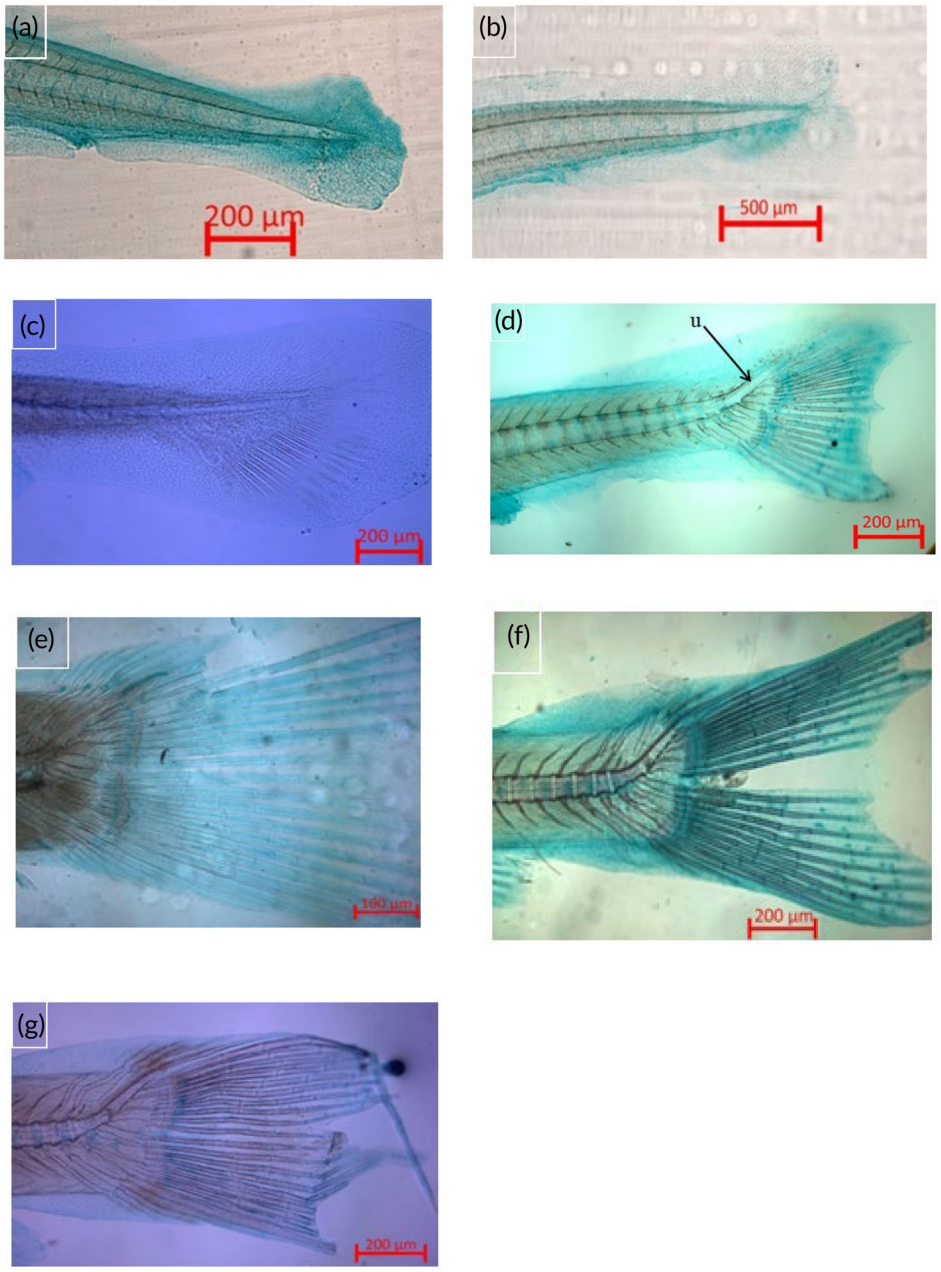

FIGURE 5 Ossification of caudal part in *Labeo calbasu* under a light microscope. u: urostyle. (a) 2 dph, 6.6 mm; (b) 4 dph, 7.08 mm; (c) 6 dph, 8.47 mm; (d) 7 dph, 9.32 mm; (e) 11 dph, 11.52 mm; (f) 15 dph, 13.73 mm; (g) 20 dph, 15.6 mm

total hardness were found in optimum range during the broodstock and larval rearing (Table 2).

4 | DISCUSSION

Skeletal staining is an important method in the anatomical study of bone development of any vertebrate. Different protocols have been proposed since 1897 for staining of cartilages; nonetheless in 1941, Toluidine blue was introduced for staining cartilaginous parts of the skeletal system, and later it was used with Alizarin red to cause differential staining (Aliesfehani, 2015). In 1970, Alcian blue was used for staining the cartilaginous parts of the skeletal system, and subsequently, the double skeletal staining procedure using Alizarin red and Alcian blue has been widely used (Aliesfehani, 2015). In this study, the red fluorescence emission of the calcium-binding fluorescent dye Alizarin (1,2-dihydroxyanthraquinone), when excited with green light, was used in imaging the early and late stages of skeletal ossification in *L. calbasu*. The skeletal ontology of *L. calbasu* corroborated with other teleost fishes like *Prochilodus lineatus* (Hernández *et al*., 2016), *Sebastes koreanus* (Yu & Kim, 2016) and *Danio rerio* (Bensimon-Brito *et al*., 2016). The ossification of cranial bones and vertebral column started on 2 and 6 dph, respectively, and completed between15 and 20 dph with the complete development of the caudal skeleton. In line with the present study, all vertebrae showed complete ossification along with the interneural and interhemal spines by

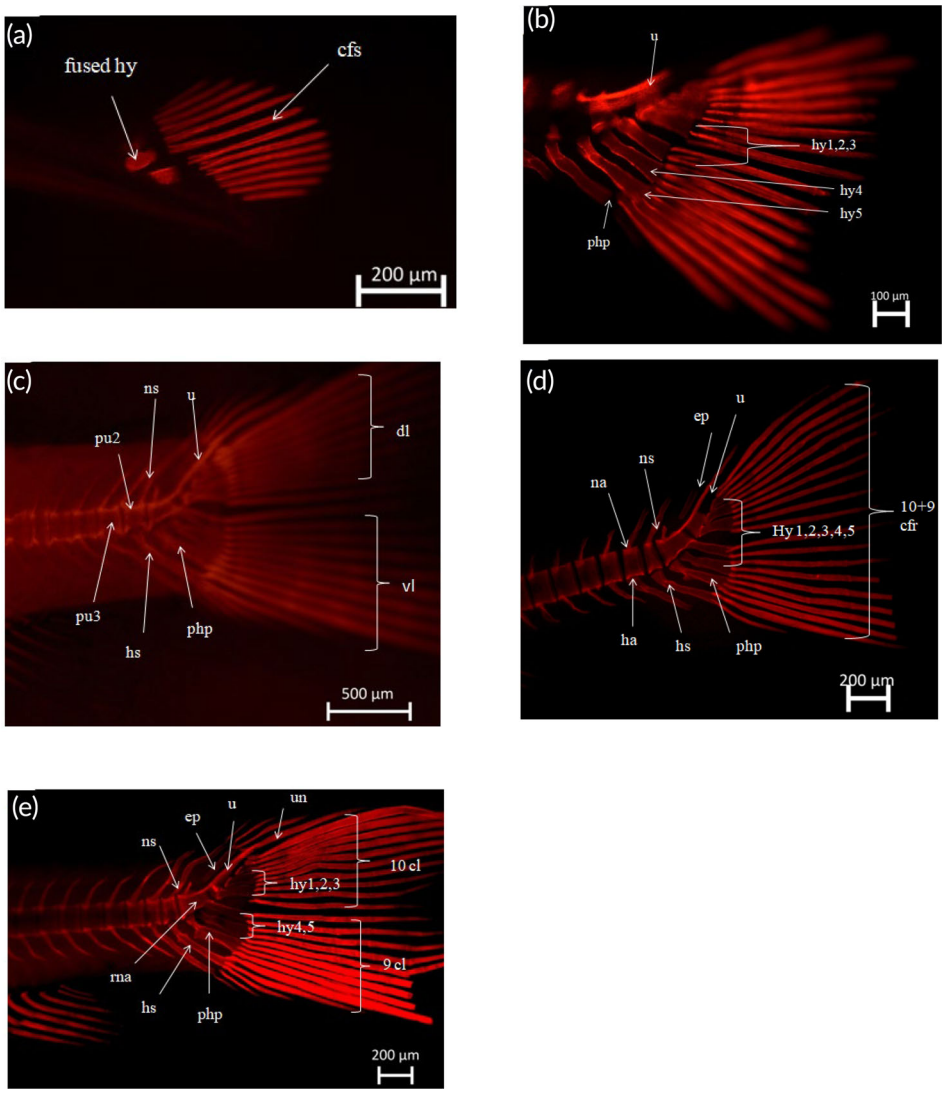

FIGURE 6 Ossification of caudal part in *Labeo calbasu* under a fluorescent microscope. (a) 6 dph, 8.47 mm; (b) 7 dph, 9.32 mm; (c) 11 dph, 11.5 mm; (d) 15 dph, 13.7 mm; (e) 20 dph, 15.6 mm; hy: hypurals; cfs: caudal fin spines; php: parhypurals; ns: neural spine; hs: hemal spine; dl: dorsal lobe; vl: ventral lobe; na: neural arch; ha: hemal arch; ep: epural; u: urostyle; cfr: caudal fin rays; rna: Rudimentary neural arch; pu2: first preural; pu3: second preural; un: uroneural; cl: caudal lepidotrichs

TABLE 2 The average water quality parameters of broodstock and larval rearing tank

Parameters	Broodstock rearing tank	Larval rearing tanks
Water temperature (°C)	27.9 ± 0.50	28.4 ± 0.29
pH	7.87 ± 0.18	7.63 ± 0.10
DO (mg l^{-1})	6.51 ± 0.25	6.24 ± 0.14
Free CO_2 (mg l^{-1})	0.95 ± 0.30	1.2 ± 0.17
Conductivity (µS cm^{-1})	318 ± 6.25	330.0 ± 1.60
Total alkalinity (mg l^{-1})	118.30 ± 3.89	110.0 ± 1.20
Total hardness (mg l^{-1})	152.20 ± 1.16	176.33 ± 0.52

Note: n = 5. Values are mean ± S.E. S.E., standard error.

10–18 dph in *Liobagrus obesus* (Seo *et al*., 2018). In *L. calbasu*, the ossification of the hyomandibular, frontal, lateral ethmoid, parasphenoid and the opercle takes place within first 6 days after hatching. The branchiostegal rays, the lower jaw with its angular and articular bones, and the upper jaw with its maxillary and premaxillary were distinct by 7 dph. By the end of 11 dph, cranial bones such as frontal, sphenotic, palatine, opercular bone were more articulated. The ossification of pectoral girdle bone with clavicle and scapula was marked at the end of 15 dph, and the ossification of the pectoral fin rays was prominent by 20 dph. The development of cranium and the associated bones in *L. calbasu* is similar to those in *L. obesus* (Seo *et al*., 2018) which indicated simultaneous ossification of parasphenoid, jawbones and clavicle supporting pectoral fins.

With the larval growth, the notochord was gradually replaced by ossified vertebrae which provided stronger attachment sites for the muscles. The progressive ossification of the vertebral centra proceeded from the abdominal to the caudal vertebrae, and the urostyle was fully ossified just before the completion of the ossification of the caudal vertebrae. The spines of the dorsal, pelvic and the anal fins along with the interneural and the interhemal spines began and completed their ossification on 11 and 20 dph, respectively.

In some Cyprinids like *Labeo*, the last 4–5 vertebrae participate in the formation of the caudal skeleton, which supports the caudal fin made up of the first and second preural, urostyle, 5 hypurals, parhypurals and the epurals, which start to ossify by 7 dph and complete by

20 dph (Yadav *et al*., 2018). Rojo (2017) reported that the epural bone is remnant of the neural spine of the last vertebrae. In actinopterygian fishes, serial cartilages bones found in epaxial region of caudal fins are termed as epurals, which elongate into rods of cartilage and notochord flexes dorsally during ontogeny (Doosey & Wiley, 2015). The hypurals and parhypural not only support the caudal rays but also provide a mean for differential movements between the upper and lower parts of the fin base (Gosline, 1997). The neural and hemal spines emerging from the diurals (PU2 and PU3) also support the lepidotrichia. The lepidotrichia in *L. calbasu* is in 10 + 9 arrangement with simple and principal branched rays, and its ossification completes by 20 dph. *L. Calbasu* displayed an advanced form of caudal skeletal complex even though there are species-specific variations in caudal skeleton of Cyprinid fishes (Yadav *et al*., 2018).

Water quality management determines the success rate of larval rearing; thus, an optimum range of water parameters needs to be maintained for breeding as well as embryonic and larval development. Growth, larval development and swimming performance in fish have distinctive responses to change in environmental parameters (Green & Fisher, 2004). Environmental change can cause shifts in the rate of ontogenetic changes (Koumoundouros *et al*., 2002). The optimum water quality parameters like temperature 24–30°C, pH 6–9, DO 5–6 mg l^{-1}, free CO_2 <8.0 mg l^{-1}, total alkalinity 60–300 mg l^{-1} and total hardness >40 mg l^{-1} is required for Indian major carp larval development (Ayyappan *et al*., 2006). The water quality parameters of the present study are conducible for larval rearing of *L. calbasu*.

5 | CONCLUSION

The present ontogenic study of skeletal system of the larval stage of *L. calbasu* will help in the better taxonomic positioning of the species. It will also help in assessing the swimming behaviour of the species during its larval stage. Furthermore, understanding the ossification process of the jaw and mouth bones of the growing larvae would be helpful to understand the food and feeding habit of the larvae. The earlier completion of the skeletal ossification has the better chance of the larval survival owing to the better swimming and prey capture ability. As the skeletal ossification of *L. calbasu* completes by 20 dph, a drastic reduction in the larval mortality is expected at this stage. The outcome of the present study, in turn, can be useful for breeders and nursery growers.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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