Growth of the Medaka (VIII) − Develpoment of Pharyngeal Bones with Teeth during Metamorphosis.

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ABSTRACT

The present report is to describe the morphological changes in developing pharyngeal bones with teeth in the period of metamorphosis in the medaka, *Oryzias latipes* and to contribute toward providing anatomical data of the medaka as an excellent model for taxonomic and evolutionary analysis. The developmental process of the tooth ridges and the skeletal elements was morphometrically examined in metamorphic phases from the larval to the juvenile. When fry hatch, the lower (fifth ceratobranchials, Cb5) and upper (third pharyngobranchials, Pb3) pharyngeal bones that are closely apposed are already observable. The size of the Cb5 and Pb3 bones increases progrssively in parallel with ossification in proportion to body growth. Shortly after hatching, the teeth in these pharyngeal bones already erupt on the ossified thin toothplate. The first tooth ridge with 6-7 teeth of the primary toothplate in these pharyngeal bones forms in larvae about 5.0 mm total length (TL). The tip of each conical tooth is posteriorly curved. When larvae grow 7.1 mm TL, second pharyngobranchials (Pb2) apposing to the anterior region of Pb3 can be detected. Newly erupting teeth in the posterior side of the preexisting tooth row can be detected in the upper and lower pharyngeal bones. The number of tooth ridges on the Pb3 toothplate increases linearly until 10 mm TL in proportion to body size but thereafter adds gradually. The present anatomical study appears to indicate that pharyngeal bones become nearly complete by about 15 mm TL.

Keywords: pharyngeal skeleton, tooth ridge, metamorphosis, medaka

INTRODUCTION

So far, there are many investigations on the partial or whole morphological observations of the head skeletons in the adult medaka (Kulkarni, 1948; Rosen, 1964; Yamamoto, 1975; Iwamatsu and Hirata, 1980; Rosen and Parenti, 1981; Yabumoto and Ueno, 1984; Werneburg and Hertwig, 2009). The tooth structure of the jaws in the medaka also has been examined morphologically in different species (Parenti, 1987). Specifically, Parenti (1987) reported the comparative anatomical data on the structure and development of the oral and pharyngeal jaws along with systematic inferences on the evolution of advanced teleost fishes including medaka. The embryonic and post-embryonic development of the whole head skeletons including oral regions has also been morphologically examined in the medaka, *Oryzias latipes,* by Langille and Hall (1987, 1988; Werneburg and Hertwig, 2009). However, little attention has been paid to development of pharyngeal bones. To obtain a detailed knowledge of the dynamics of the tooth pattern formation in odontogenesis, the time and order of development of pharyngeal bones must be precisely examined. The anatomical data of developing pharyngeal skeletons in the growing period from larva to young fish during metamorphosis may be used to answer intriguing questions on the relationship between morphology and function of adult pharyngeal bones with teeth.

Recently, the genetic pathways involved in development of the oral and pharyngeal dentition have been investigated in the medaka (Atukorala *et al*., 2010/2011; Mantoku *et al*., 2016), of which genome has been almost fully sequenced. Atukorala et al. (2010/2011) confirmed, in the *edar* mutant medaka fish that is referred to as reduced scale-3 (rs-3), the differential involvement of Eda-Edar signaling in the induction and growth of scales and teeth of pharyngeal odontogenesis. It has also been suggested that *eve 1* (Debiais-Thibaud et al., 2007) and *Sox 2* (Abduweli et al., 2014) express in each tooth family to maintain continuous tooth formation and replacement in the pharyngeal dentition of the medaka. Thus, despite the increasing genetic and developmental information, detailed anatomical data still are required to understand a close relationship between the architectural gene expression and visual organization of the pharyngeal dentition. The anatomical description will also contribute to provide indispensable data for the analysis of gene expression which cannot be interpreted without a detailed information on the developmental process during morphogenesis. As a matter of course, the function of pharyngeals cannot be interpreted adequately without a morphologically detailed information on the spatiotemporal order of development.

At present, the development of both oral and pharyngeal teeth in *O. latipes* has been reported by Debiais-Thibaud et al. (2007), who recommended the medaka as an excellent model to develop further insights into the evolution of odontogenesis in gnathostomes. However, the precise morphological changes in developing pharyngeal bones with teeth have not been traced throughout metamorphosis. Therefore, the present study aims at observing and describing the developmental process of upper and lower pharyngeal bones during metamorphosis.

MATERIALS and METHODS

The medaka fish, *Oryzias latipes* (d-*rR* strain), used in the present study were reared in a rectangular glass aquarium (60x35x30 cm, about 60 liters of water) under reproductive conditions (L14, D10; 26-28℃). During the rearing period, fish were fed a balanced diet (see Iwamatsu, 2014). Under these conditions, mature females mate with males and spawn 20-30 eggs every morning. After females were netted, fingers were used to pluck off a cluster of fertilized eggs that were hanging by chorionic filaments from their urogenital pores. Fertilized eggs were placed in petri dishes with water (26-27 ℃) containing 0.0002 % methylene blue to prevent fungal growth (Langille and Hall, 1988). After hatching, larvae were reared by being fed the balanced diet in rainwater containing green algae in a polycarbonate rat cage (26-28℃).

For skeletal preparation, fish were sacrificed by overdose of the anesthetic (a mixture of saturated phenylurethane solution 7: ethanol 3). After total length (TL) of deeply anesthetized fish was measured, larvae and juveniles were treated with 0.5 % NaOH containing 0.5 % formalin for a few days at room temperature, prior to whole skeletal staining. The NaOH-treated specimens were adequately rinsed in water, and then stained with a diluted alizarin red solution (0.01 % alizarin red S: Nakarai Chemicals Ltd., Kyoto) for a few hours, and an excessive staining was destained by rinsing repeatedly in water. Then, the stained specimens were finally cleared in 50 % glycerol for observation and morphometry. Skeletons were observed and measured using a stereoscopic microscope (Olympus SZX12) equipped with a calibrated ocular micrometer. Growth stages of fish were assigned following the developmental criteria and according to the total length (TL: rostral-caudal length) (Iwamatsu, 1994, 2004; Iwamatsu et al., 2003). The mean values of each number of tooth ridges in pharyngeal bones examined in growth period were represented graphically. Names used for skeletal structures follow Owen (1984), Yabumoto and Ueno (1984) and Parenti (1987).

OBSERVATIONAL RESULTS and DISCUSSION

As seen in the head skeleton of the medaka 5.0 mm TL shortly after hatching (Fig. 1), the larvae already possess the upper and lower pharyngeal bones that occupy the position in front of the short esophagus. According to Debiais-Thibaud et al. (2007), the first appearance on the dorsal pharyngeal teeth is recognized in the form of a tooth bud around developmental stage 30 (28℃). At stage 32, three tooth buds without alizarin red staining are detectable. By stage 38 (8 days post-fertilization) the upper pharyngeal (third pharyngobranchial: Pb3) has five dental caps with alizarin red staining. Rosen and Parenti (1981) first found the absence of a fourth pharyngobranchial element as an atherinomorph synapomorph. Debiais-Thibaud et al. (2007) describes the upper pharyngeal toothplate as the fourth pharyngobranchial (Pb4) but not the third pharyngobranchial (Pb3). Thereby,

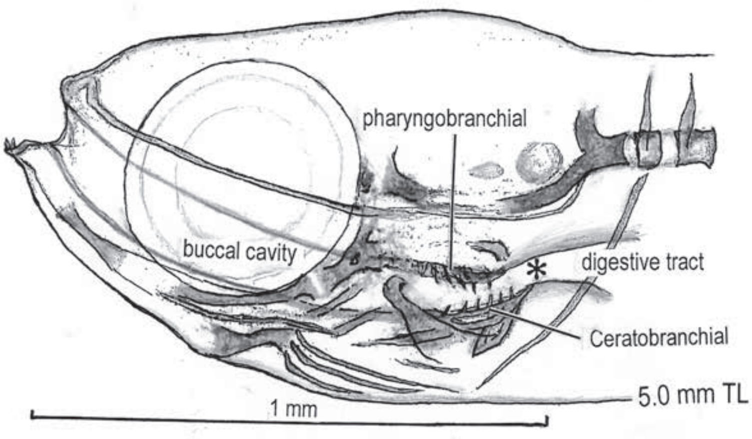

Fig. 1 Schematic diagram of the head region of an early *Oryzias latipes* larva.
In this lateral view, pharyngeal bones and teeth are located at the posterior end of the pharynx in the early larva (about 5 mm TL). This is a figure modified from Fig. 3A in Iwamatsu (2021). The asterisk indicates the esophageal region of the digestive tract. Anterior is to the left.

"fourth pharyngobranchial" has been proposed to be a misidentification of Langille and Hall (1987: Figs. 8 and 9 in 144 and 146 pages), as pointed out by Parenti (1993). The proposition is supported by many investigations in *Oryzias* species such as *O. carnaticus*, *O. matanensis*, *O. profundicola* (Parenti, 1993, 2008), *O. celebensis* (Iwamatsu and Hirata, 1980) and *O. latipes* (Yabumoto and Ueno, 1984; Iwamatsu and Hirata, 1980). Therefore in the present study, the term of Pb3 is used.

Development of pharyngeal skeletons after hatching

In *O. latipes*, as described in the present observation of young fish and previous anatomical studies of adult fish (Iwamatsu and Hirata, 1980; Parenti, 1987), the lower pharyngeal (fifth ceratobranchial: Cb5) and Pb3 bones are closely apposed, but not fused respectively. Among other beloniform fishes, the needlefishes and sauries have the upper pharyngeal bone to be closely apposed and the lower pharyngeal bones to be fused. And otherwise, in halfbeaks and flying fishes, both lower and upper pharyngeal bones are fused (Parenti, 1987).

At the time of hatching, the upper and lower pharyngeal bones have conical denticles on incomplete rod-like cartilages (rd), as seen in Fig. 2. The pharyngeal teeth help the pharynx in swallowing captured food into the esophagus. Skeletal development of the pharyngeal regions occurs continuously after hatching. The conical teeth point their tips toward the pharynx. In the early larva (ca. 4.5 mm TL), ossification stained with alizarin in several tooth buds is ascertained on the rod-like cartilages of the upper Pb3 and the lower Cb5 bones. Erupting new teeth are located at the rostral side apart from these bones (Fig. 2A). The Pb3 bones show the rod-like cartilagious toothplate with a modicum of gap space. The early primary toothplates of Pb3 are thin bones with the rod-like cartilages. Shortly after the initiation of ossification of the toothplate, a blank space appears at a central region of the toothplates. Thus, the first tooth ridge forms by separating from the toothplate. Teeth (5-8 in number) in Pb3 and Cb5 bones erupt in larvae of 4.3-4.6 mm TL soon after hatching.

When larvae grow to about 4.5 mm TL (about 9 days post-fertilization), the new tooth ridge with 6-7 teeth forms in toothplates of the ossified left and right Pb3 (Fig. 2A), in accordance with Pb4 in Debiais-Thibaud et al. (2007). In the first tooth row of the toothplate in Cb5, 4-5 teeth are observed in the anterior side of the plate (Fig. 2A).

When larvae reached 6 mm TL, Pb3 displayed 2-3 tooth ridges, although Debiais-Thibaud et al. (2007) has illustrated Pb3 equipped with 5 tooth ridges of the larvae which reached 6 mm in length. However, when TL of larvae becomes about 5.0 mm (Fig. 2B), the first tooth ridge with 6-7 conical teeth forms in the Pb3 , as well as Cb5 in which a flat projection is seen beneath the ventral surface of the toothplate. In adult fish of *O. latipes* and *O. celebensis* (Iwamatsu and Hirata, 1980) the flat projection

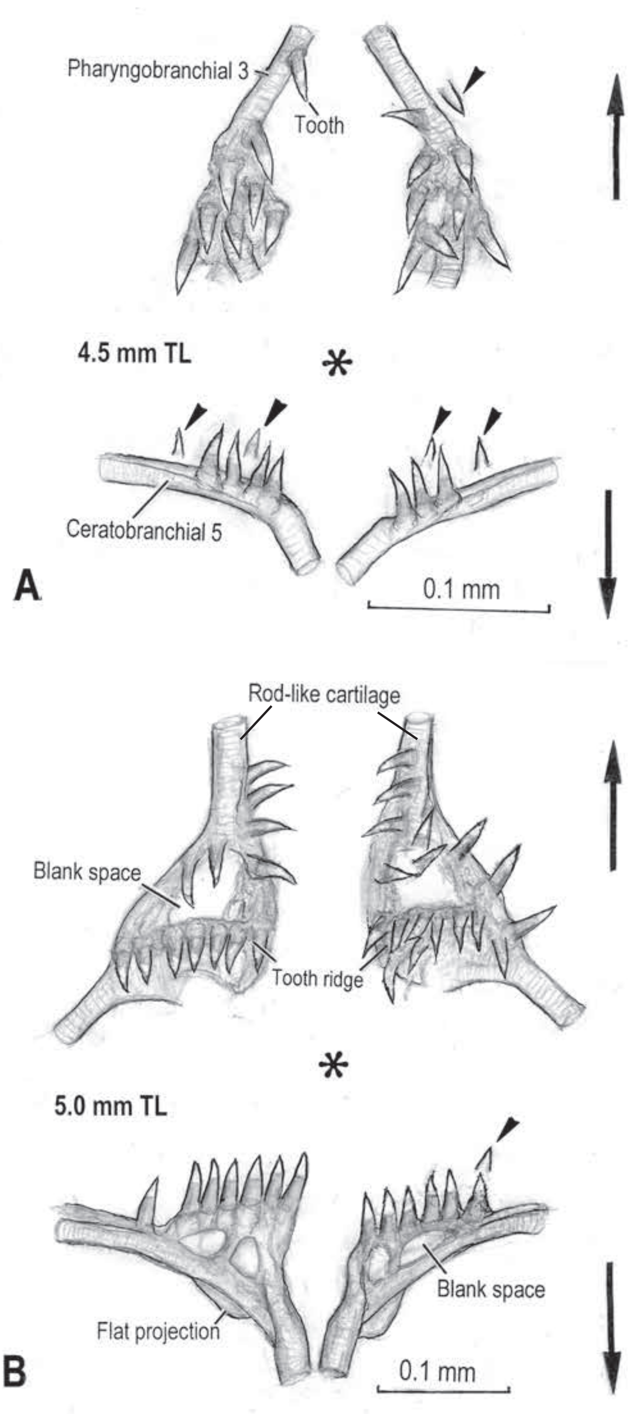

Fig. 2 Occlusal views of the pairs of upper and lower pharyngeal bones in *Oryzias latipes.* The left and right pairs of the lower (dorsal view) and upper (ventral view) pharyngeal bones (A: 4.5 mm TL, B: 5.0 mm TL) possess conical teeth. Newly erupting teeth (arrowheads) locate in posterior side apart from the attachment bone. Each asterisk indicates the connecting to the esophagus (digestive tract). Large vertical arrows show the rostral direction.

turns toward laterally along by 3 or 4 tooth ridges. The retractor muscle of Cb5 firmly attaches in the anterior region of this projection. In the right and left Pb3 toothplate with the rod-like cartilage, a new tooth is also seen on the first tooth ridge that is individualized from the anterior arch of Cb5 (Fig. 2B). A blank space is observable in respective pharyngeal bones of Pb3 and Cb5 (Fig. 2B). Thus, in larvae 5.0 mm TL, the first ridge forms in Pb3 and Cb5. The second pharyngobranchials (Pb2) is still not detectable in the anterior region of Pb3, prior to formation of the first tooth ridge in larvae about 5.0 mm TL. According to Debiais-Thibaud et al. (2007), the second tooth ridge of Cb5 forms when larvae reach about 6 mm TL, and the third tooth ridge starts to develop at about 8 mm. However, in the present study, Cb5 remains to have the first tooth ridge (first single row of teeth) in larvae 7.1 mm TL (Fig. 3).

In the larva of about 7 mm TL the three tooth ridges with 3-8 teeth form in the toothplate of Pb3 (Fig. 3). The length of these bones increases laterally in proportion to the body length. In Cb5 with a single tooth ridge laterally extended, blank spaces are recognized between the Cb5 bone with the flat projection and tooth ridge with only 8-9 teeth. And then, the poor Pb2 with a few conical teeth appose in the outer sides of the anterior regions of left and right Pb3 bones. The fourth epibranchials are for the first time detectable in the anterior region of Pb3. New teeth are observable apart from the posterior side of the first tooth ridge.

In the early juvenile (about 10-15 mm TL) in which all elements of pharyngeal bones appear but sexually immature (Langille and Hall, 1987), Pb3 and Cb5 differentiate swiftly. By the end of early juvenile phase (10.8 mm TL), the number of tooth ridges is 5-6 in Pb3, and 3 in Cb5 (Fig. 4). Newly erupting teeth are also found in the posterior side of tooth ridges. The conical teeth on different-sized tooth ridges are varied from 3 to 20. Third epibranchial is detectable apart from the outside of Pb3.

In juveniles 15.1 mm TL, the 8th tooth ridge forms in Pb3 (Fig. 5). The irregular rows of caniform teeth on 3-4 tooth ridges in Cb5 are observed. The Pb2 bones tightly attaches itself to Pb3 bones by a connecting cartilage on the adjacent side of these bones.

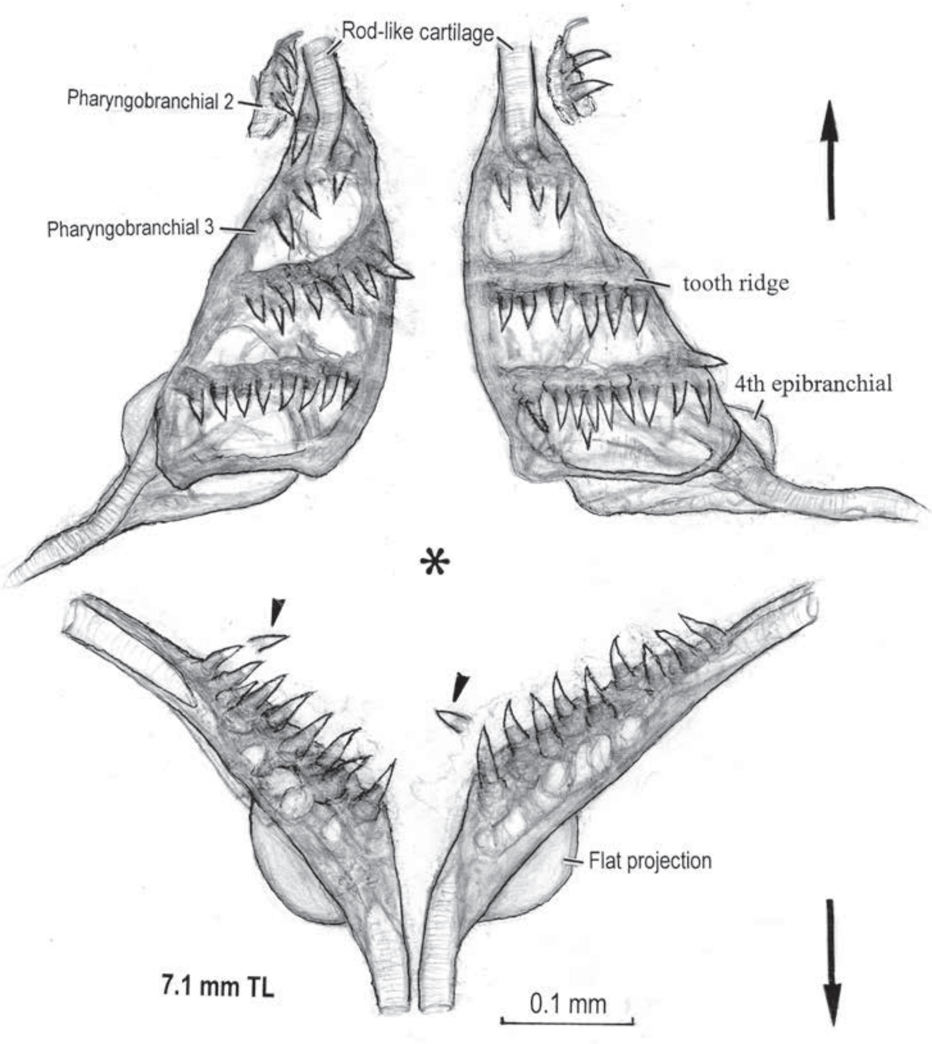

Fig. 3 Occlusal views of pharyngeal bones in *O. latipes* larva 7.1 mm TL.

Change in the number of tooth ridges upon growth

The number of tooth ridges with conical teeth on both bones of Pb3 and Cb5 increases as the body of the medaka grows and becomes linearly in proportion to body size by the early period of metamorphosis (Fig. 6). According to Parenti (2008), an immature male (11.5 mm standard length) has 3 complete tooth rows. New tooth ridges of Pb3 are progressively added on the anterior margin of preexisting pharyngeal toothplate by 10 mm TL, and the number of tooth ridges is 2-3, in consistent with her observation. In young fish about 15 mm TL, the number of tooth ridges is 3-4 in Cb5 and 8 in Pb3 (Fig. 6). The subsequent increase of tooth ridges becomes somewhat slow in young fish more than 15 mm TL near the period of sexual maturation (Fig. 6). The fact that newly erupting teeth are always located in the posterior side may be involved in the place where the osteoblasts gather (Mantoku et al., 2016)

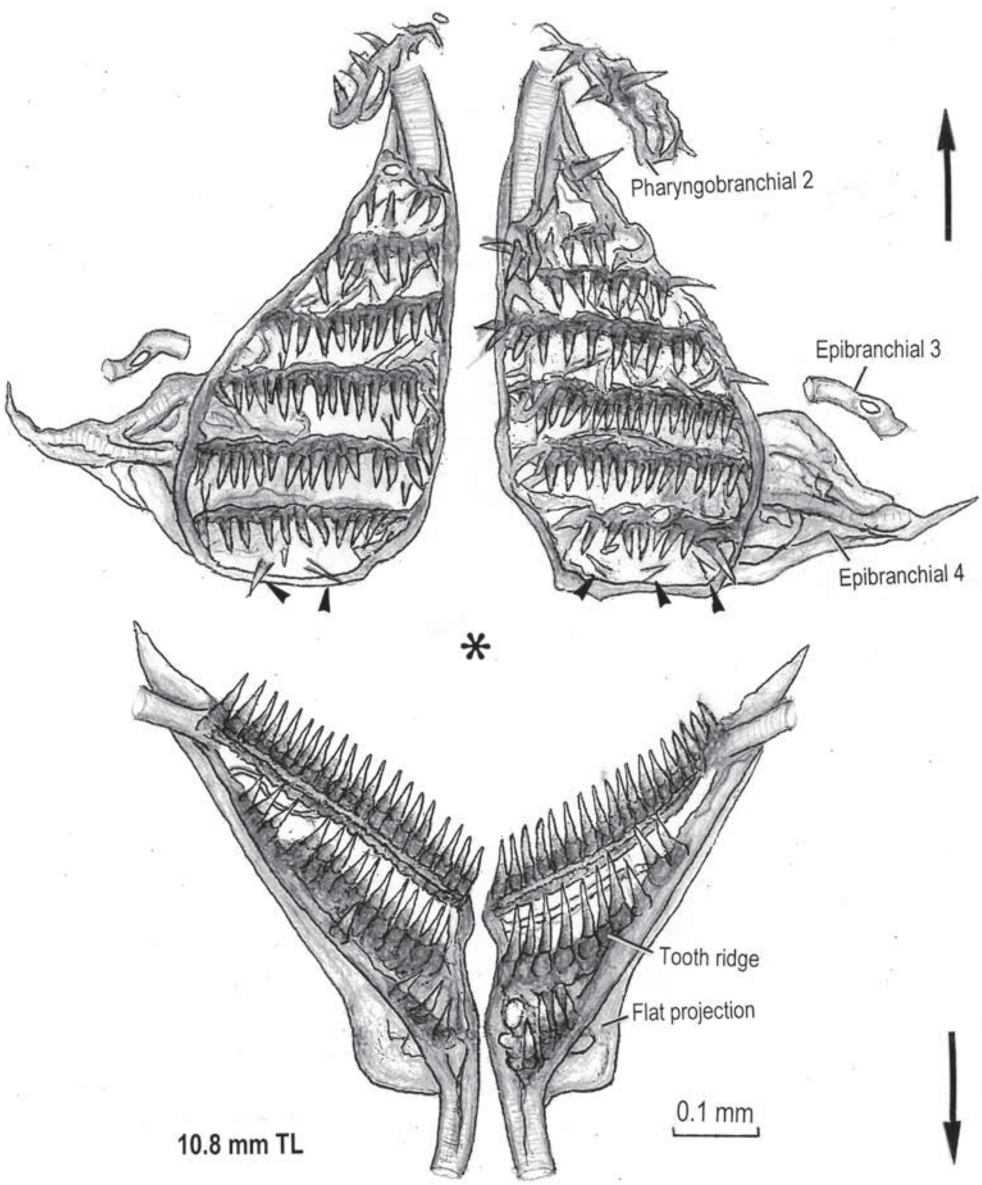

Fig. 4 Occlusal views of pharyngeal bones in *O. latipes* juvenile 10.8 mm TL.

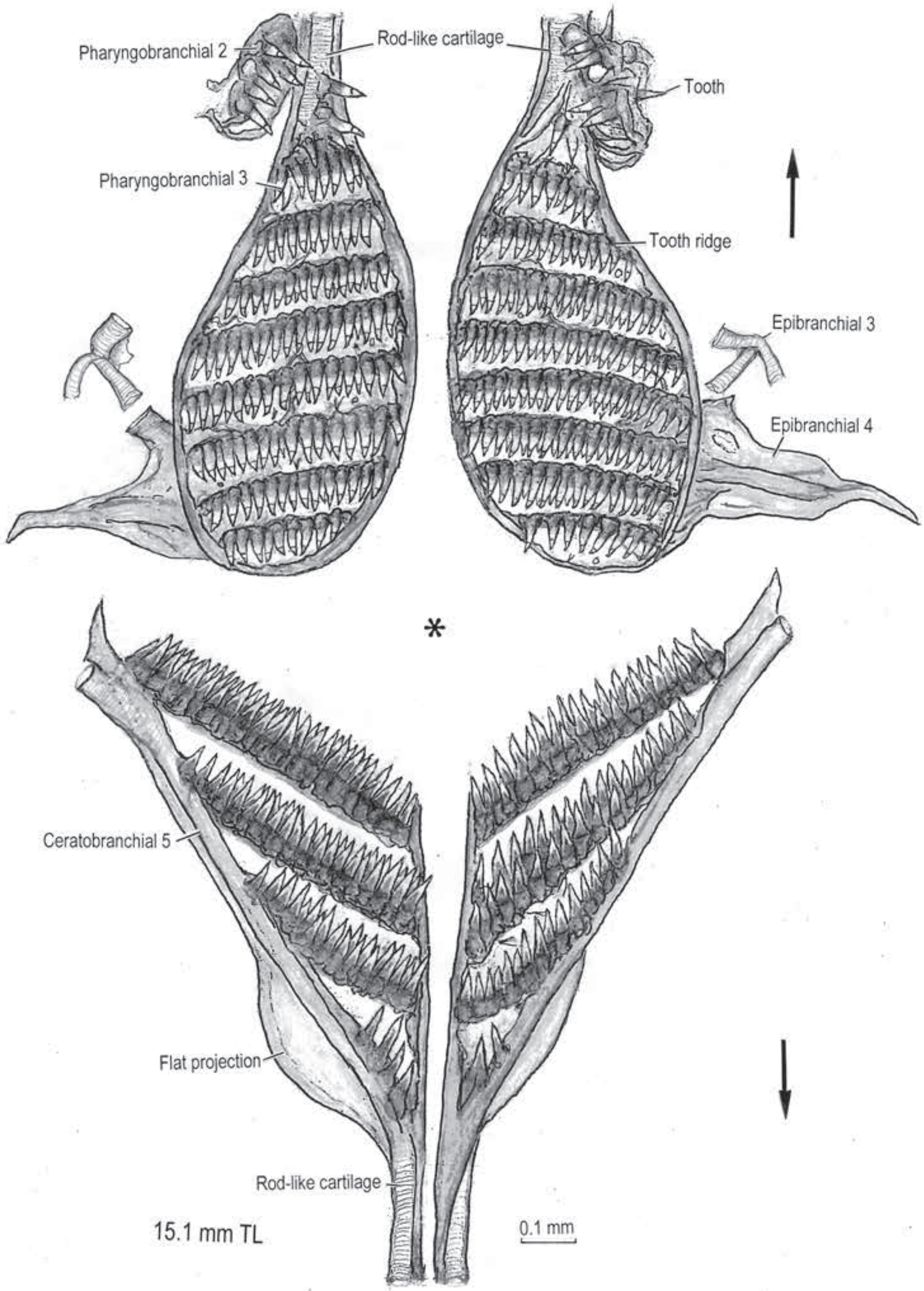

Fig. 5 Occlusal views of pharyngeal bones in *O. latipes* young fish 15.1 mm TL.

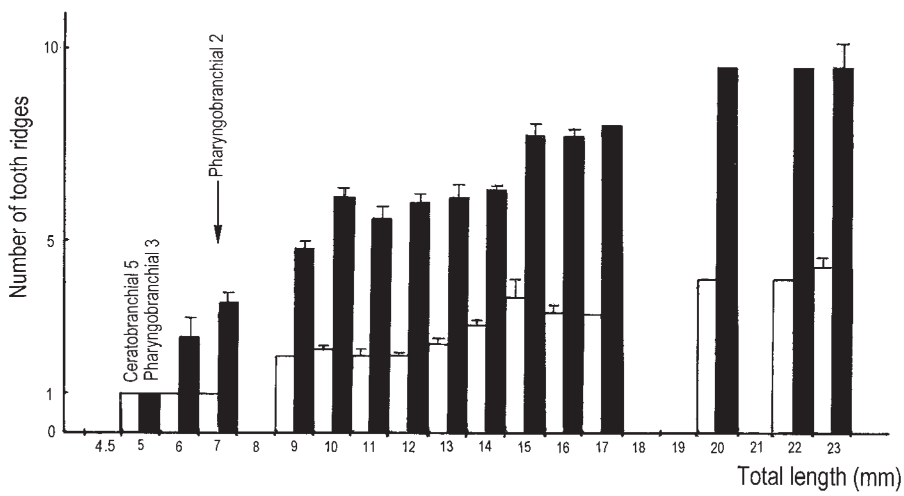

Fig. 6 Change in number of tooth ridges of pharyngeal bones during metamorphosis of *O. latipes*.

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