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Classification of Late Embryonic Stages of Medaka, *Oryzias latipes*

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Abstract To indicate more clearly the stages of development in late embryo of the medaka, *Oryzias latipes*, the quotients obtained from the length of tail (measured from the vent to distal end of caudal fin) in microns divided by 100 μm were adopted to nominate stage numbers. The 25 stages thus defined were checked against the calcification of 8 elements in the chondrocranium, and also 4 other structures which develop numerically. By the observations of development (cartilages and calcification indicated by alizarin red S staining) of the 8 elements—parasphenoid, clavicle, operculum, occipital arch, hyomandibular, ceratohyal, anterior otic process and mandibular, the stages defined here were found to classify the order and degree of development of bony elements more precisely than other stage classification in the past. The numerical structures—branchiostegal rays, pharyngeal teeth, vertebrae and mandibular teeth also demonstrated clearly their development corresponding to tail length. The "critical" stages in embryonic development of the medaka as shown by the length of notochord with vacuolized cells associated with development of bony elements were also noted.

Matsui (1949) made an excellent description of normal development of medaka embryos, and the stages established by him have been used by many investigators ever since. Although the late embryonic stages after the beginning of heart beat (Matsui's stage 24) range three quarters of the total embryonic period, Matsui (1949) divided the period into only nine stages. The stages defined by him are not necessarily clear, and one stage includes many developmental changes. Matsui's classification was later modified a little by Gamo and Terajima (1963), based on their observations of intact embryos. A trial of a more precise classification of late embryos is presented in this paper.

The number of myotomes is one of the useful criteria for the determination of a given stage, but it could be adopted for only middle embryonic stages, because the increase of myotomes after Matsui's stage 27 is too small. The total length of an embryo, another measurable character, carries difficulty in precise measurement due to cephalic flexure. In contrast, the tail length measured from the vent to the distal end of the caudal fin is believed to present a more precise and easier to use measurements for determining a stage of late embryos, at least in the medaka. The tail grows to the length of 2,500 μm after Matsui's stage 24 until the time of hatching. Furthermore, it more closely corresponds to the morphological and biochemical changes taking place in the body of an embryo. To evaluate the significance of the stages thus defined, the calcification of 8 elements in the chondrocranium was observed corresponding with these stages. Also, the development of 4 numerical structures was tested in the same way.

Material and method

Orange-red medakas, *Oryzias latipes*, obtained from Yatomi near Nagoya City were kept outdoors and fed with a diet of toasted whole barley flour mixed with shrimp powder. The spawned eggs were gathered in the morning and allowed to develop in a balanced salt solution. Egg chorion was removed by needles under the microscope and the tail length was measured by occular micrometer. The tail begins to develop at Matsui's stage 24 when the heart begins to beat, and the length of tail reaches about 2,500 μm at hatching (Matsui's stage 33). During these late embryonic stages (Matsui's stages 24–33), the length of tail defined as above was measured, and the length expressed in microns was divided by 100 μm. In the calculation of quotients, fractions of more than 0.5 were counted as one and the rest were eliminated. Through such a treatment on 500 embryos of late developmental stages, there were established 25 stages (Table 1).

The chondrocrania of embryos were stained by 0.1% alizarin red S in 0.05% NaOH for 20

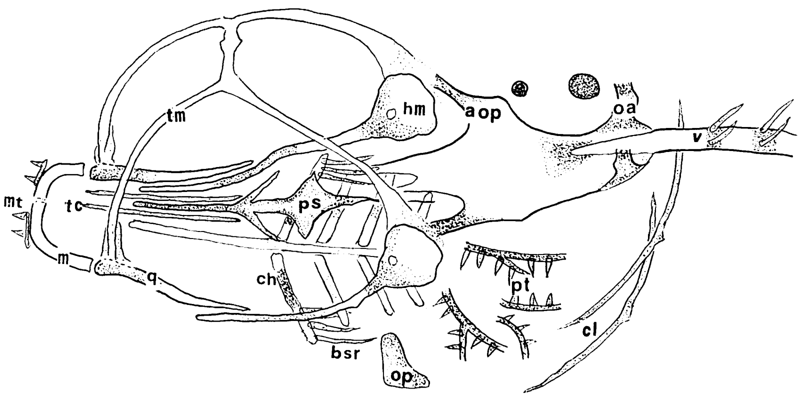

Fig. 1. Stereogram of chondrocranium of embryo in late developmental stage of *Oryzias latipes*. The dotted part shows calcified bone, aop, anterior otic process; bsr, branchiostegal ray; ch, ceratohyal; cl, clavicle; hm, hyomandibular; m, mandibular; mt, mandibular teeth; oa, occipital arch; op, operculum; ps, parasphenoid; pt, pharyngeal teeth; q, quadrate; tc, trabecula cranii; tm, taenia marginalis; v, vertebra.

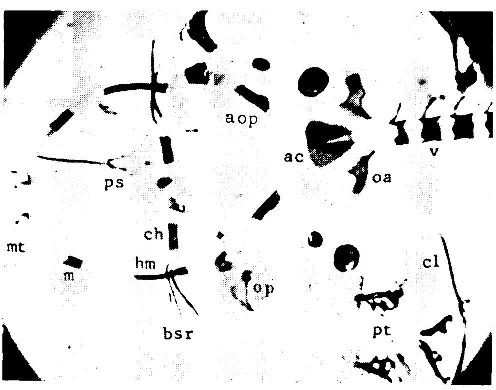

Fig. 2. Bony elements in chondrocranium of an embryo (stage 25) of *Oryzias latipes* and anterior part of vertebrae stained by alizarin red S. The parts stained dark in the picture are bones calcified and parts not stained are mostly cartilagenous. Symbols are the same as in Fig. 1.

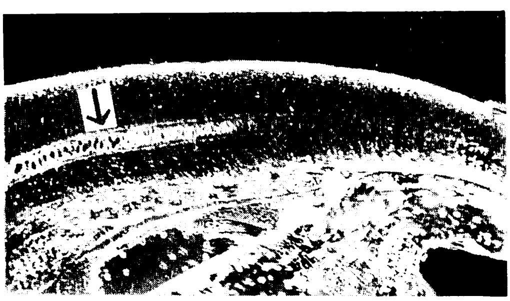

Fig. 3. An embryo of *Oryzias latipes*. Arrow shows notochord with vacuolized cells.

minutes and squeezed under a cover glass, and calcifed parts were observed (Fig. 2). The stereogram of the chondrocranium was then made from the stained samples preserved in glycerin (Fig. 1). Each portion of the chondrocranium and its neighbouring structures were termed according to Berrill (1925), De Beer (1928), Goodrich (1930) and Yabumoto and Uyeno (1984). Although the cartilages of the chondrocranium before calcification were visible without staining and were enough to understand the degree of the embryonic development, the alcian blue staining method of Dingerkus and Uhler (1977) was also used. Since the quantitative measurement of calcification is difficult, merely empirical evaluation was attempted in the present experiment, in which about five hundred embryos were used.

Results

The significant changes and developments in structure and function of some organs which were observed in the present study are listed below by the stages derived from the tail length. The appearance and calcification of 8 elements in the chondrocranium are tabulated (Table 1) to show a definite correlation of development of each element with stages. The same table also shows the increase of numerical structures corresponding with stages.

Stage 1. Kuffer's vesicle remains. 15 myotomes. Heart commences pulsation.

Table 1. Development of bones in chondrocranium and number of calcified pieces in 4 numerical structures arranged by the developmental stages expressed by tail length. − sign indicates cartilaginous development, and + sign the calcification. (−) sign indicates bone which is not calcified. Cartilages do not develop well in parasphenoid and clavicle. The degree of calcification is shown by the numbers of + signs. Asterisk indicates notochord with vacuolized cells. Developmental stages proposed by Matsui (1949) and Gamo and Terajima (1963) are also presented for comparison.

Stage in tail length (original)	Stage by Matsui (1949)	Stage by Gamo and Terajima (1963)	Length of notochord (100 μm)*	Bony elements								Number of calcified pieces			
				Parasphenoid	Clavicle	Opercula	Occipital arch	Hyomandibular	Ceratohyal	Anterior otic process	Mandibular	Branchiostegal rays	Pharyngeal teeth	Vertebrae	Mandibular teeth
1	24	24	0												
2	25	25	0												
3	—	—	0												
4	26	26-1	5												
5	—	—	7												
6	—	26-2	8.5												
7	—	—	10.5												
8	27	27	12												
9	—	—	13.5												
10	—	—	15.5												
11	—	—	17.5												
12	28	28	19												
13	—	—	20												
14	—	—	22	(−)	(−)										
15	29	29	23	(−)	(−)	−	−								
16	—	—	24.5	(−)	(−)	−	−		−						
17	—	—	26	+	+	−	−	−	−			2	2		
18	30	30	27	++	++	+	−	−	−			2	4		
19	—	—	28	+++	+++	+	+	−	−	−		2	8	8	
20	31	31	29	+++	+++	++	++	−	−	−	−	2	10	12	
21	—	—	30	+++	+++	++	++	+	−	−	−	2	12	14	
22	32	32	31	+++	+++	+++	+++	+	−	−	−	4	14	16	
23	—	—	32	+++	+++	+++	+++	++	−	−	−	4	16	20	
24	33	33	33	+++	+++	+++	+++	+++	+	+	−	4	20	24	2
25	—	—	34	+++	+++	+++	+++	+++	++	++	+	6	24	28	4

Stages 2–3. 18–22 myotomes. Blood circulation commences. Leucophore-like chromatophores appear under brain. Kuffer's vesicle disappears.

Stages 4–5. 23 myotomes. Two otoliths are seen in each ear vesicle. Melanophores appear in eye vesicles. Heart is constricted into two parts. A mass of vacuolized cells appears in the anterior part of the notochord (Fig. 3). The other part of the notochord has faint stripes and the posterior end is obscure.

Stages 6–7. 24 myotomes. Pectoral fin rudiments are formed behind ear vesicles. The length of the notochord with vacuolized cells is 0.8–1.1 mm.

Stages 8–13. No characteristic changes occur in these stages except the elongation of tail and notochord with vacuolized cells.

Stage 14. Parasphenoid and clavicles appear but they are not calcified (Table 1). Parasphenoid is between the eyes and clavicles are readily found near the tip of notochord as fine rods.

Stages 15–16. Occipital arches with characteristic cartilaginous cells appear beside the tip of notochord.

Stage 17. Parasphenoid and clavicles are stained lightly by alizarin red S, indicating the beginning of calcification. Cartilaginous cells of occipital arches and parachordal are numerous and clearly visible. A pair of calcified small branchiostegal rays and opercula are observed between otoliths and eye. Small arrow-head shaped pharyngeal teeth are visible. Some of them are not calcified.

Stage 18. The calcification of parasphenoid and clavicles increase. Opercula and branchiostegal rays are still small. The cartilage of hyomandibular and ceratohyal are visible. The number of pharyngeal teeth is 2–6 but some of them are not calcified.

Stages 19–20. Parasphenoid and clavicles are fully calcified. The calcification of occipital arches begins. Calcified opercula and branchiostegal rays are larger. Anterior otic process and teania marginalis are observed in the specimen stained by alucian blue. Some of the pharyngeal teeth are still not calcified. Several calcified vertebrae appear in the anterior part of the notochord.

Stages 21–22. The calcified area of occipital arches increases. Hyomandibulars begin to be calcified and the cartilage of the mandibular appear. Another pair of small branchiostegal rays appears. The numbers of calcified pharyngeal teeth and vertebrae increase to 12–16. All pharyngeal teeth are calcified after this stage.

Stage 23. Occipital arches are fully calcified. The cartilage of ceratohyals, anterior otic processes and mandibular develop well.

Stage 24. Hyomandibulars are fully calcified. The calcification of ceratohyals and anterior otic processes begins. The number of pharyngeal teeth and vertebrae are about 20 and 24 respectively. Two calcified mandibular teeth are on the mandibular.

Stage 25. The calcification of ceratohyals and anterior otic processes increases and that of mandibulars begins. There are three pairs of branchiostegal rays. The number of calcified pharyngeal and mandibular teeth and vertebrae are about 24, 4 and 28 respectively. Taenia marginalis is not yet calcified. Most of the embryos hatch in the following stage.

Discussion

The status of development of bony elements and numerical structures described above are presented in Table 1, which will show an intimate relation with the growth of the fish expressed in the length of the tail. It may be emphatically stated that the orderly development of bony elements in the chondrocranium is demonstrated more precisely when it is collaborated with the growth of the tail than in other ways hitherto adopted.

In the present study, the length of the notochord with vacuolized cells shows a linear relation with the length of the tail, but the line demonstrates a marked flexure at around stage 15 (Fig. 4). The biological reasonings for this flexure may not be explained before further studies. The significance of this "critical" change may also be endorsed by finding the facts that a number of bony elements in the chondrocranium show their initial development (as cartilages) during the stages of flexure and numerical structures begin to develop immediately after these stages.

Matsui (1949) described the appearance of red pigment in the head region at his stage 26. Gamo and Terajima (1963) described the chromatophores with the red pigment as xanthophores which appear on the midbrain in Matsui's stage 25. However, the brick-red chromatophores could not be

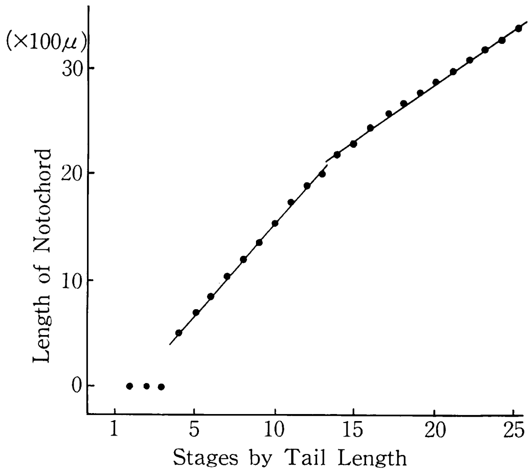

Fig. 4. The linear relationship between the tail length stages and the length of notochord with vacuolized cells. There is flexure at around stage 15.

xanthophores but could be leucophore-like chromatophores (Takeuchi 1960), and they appear at Matsui's stage 25 or tail length stages 2–3. Matsui (1949) noted that otoliths appear in stage 25 but Gamo and Terajima (1963) found that they appear in stage 26-1. In the present observations, they appear at tail length stages 4–5 which corresponds to Gamo and Terajima's stage 26-1. Similarly, the formation of pectoral fin rudiments was recorded by Matsui in stage 27 and by Gamo and Terajima in stage 26-2. In the present observation it occurs at tail length stages 6–7 which correspond to Gamo and Terajima's stage 26-2.

Hatching usually occurs at tail length stage 26 but some embryos hatch at stage 25 or 27. The numbers of both calcified pharyngeal teeth and vertebrae of newly hatched larvae count 22 to 30, which shows that hatching does not correspond to any special developmental stage of the embryo. Indeed, some embryos did not hatch at these stages but they continued to develop further.

Acknowledgments

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Literature cited

Berrill, N. J. 1925. The development of the skull in the sole and the plaice. Quart. J. Micros. Sci., 69 (274, N. S.): 217–244.

De Beer, G. R. 1928. The early development of the chondrocranium of *Salmo fario*. Quart. J. Micros. Sci., 71 (282, N. S.): 259–312.

Dingerkus, G. and L. D. Uhler. 1977. Enzyme clearing of alcian blue stained whole small vertebrates for demonstration of cartilage. Stain Tech., 52(4): 229–232.

Gamo, H. and I. Terajima. 1963. The normal stages of embryonic development of the medaka, *Oryzias latipes*. Japan. J. Ichthyol., 10 (2/6): 31–38. (In Japanese.)

Goodrich, E. S. 1930. Studies on the structure and development of vertebrates. Macmillan & Co., New York, 873 pp.

Matsui, K. 1949. Illustration of the normal course of development in the fish, *Oryzias latipes*. Japan. J. Exp. Morph., 5: 1–42. (In Japanese.)

Takeuchi, K. 1960. The behavior of carotenoid and distribution of xanthophores during development of the medaka (*Oryzias latipes*). Embryologia, 5(2): 170–177.

Yabumoto, Y. and T. Uyeno. 1984. Osteology of the rice fish, *Oryzias latipes*. Bull. Kitakyushu Mus. Nat. Hist., 5: 143–161.

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メダカ *Oryzias latipes* の胚後期の分類

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メダカの胚期の 4 分の 3 を占める胚後期（心臓搏動開始期以後ふ化まで）については，いままでに充分な分類法がなかったのでこれを試みた．尾長（肛門より尾びれ末端まで）をミクロンであらわし，これを 100 ミクロンで割って，小数以下を 4 捨 5 入した値を発生段階数とするとき，胚後期は 25 段階に分けられる．これが胚内部の変化とよく対応しているかどうかを，軟骨頭蓋の石灰化，歯の数などを例にとってしらべてみると，副蝶形骨，鰓蓋骨，後頭骨弧，舌顎軟骨の石灰化開始が，それぞれこの定義による第 17, 18, 19, 21, 24, 25 発生段階に大体おこることがわかった．咽頭歯数や石灰化した椎骨数も，大体この発生段階にしたがって増加することが観察された．

またこの発生段階数と空胞化細胞をもった脊索の長さが直線的な関係にあることがわかったが，この直線が第 15 発生段階附近で折れ曲ることが見つけられた．即ち臨界期が存在するようである．この時期以後に軟骨頭蓋

や歯の形成が観察されることと何らかの関係があるのかも知れない．

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