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Source: Zoological Science, 20(5) : 607-615

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zsj.20.607>

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Normal Growth of the “See-Through” Medaka

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ABSTRACT—The see-through stock in the medaka *Oryzias latipes*, causes pigments to be absent from the whole body and has a transparent body in the adult stage as well as during embryonic stages. To establish a standard table of growth stages for this model fish, morphological features were examined during the growing period from hatching to adulthood. The main observations were performed on morphological changes in external and internal organs that could be seen through the body wall of the living fish during growth. Finally, five growth stages from just after hatching to the adult stage were defined on the basis of synchronized or definite changes in morphology as follows: (1) stage 40 in which the nodes (joints) in bony rays of the caudal and pectoral fins first appear, (2) the stage 41 in which the ribs and the anal, dorsal and ventral fins are formed by degeneration of the membrane fin folds, as recognized by the first appearance of nodes in the fin rays of the anal, pectoral and dorsal fins, and the parallel distribution of the dorsal artery and ventral vein of the tail, (3) stage 42 in which the 2-spiral pattern of the gut, the ray nodes in the ventral fins, and the scales first appear, (4) stage 43 in which early secondary sexual characters such as urinogenital protruberances (female) and papillar processes (male) appear, (5) stage 44 in which the 3-spiral pattern of the gut and the papillar process on the 2nd ray of pectoral fins (male) appear.

Key words: growth stage, morphology, organogenesis, secondary sexual character, see-through medaka

INTRODUCTION

The development of fishes provides the most simple model for development of vertebrates. For variety of reasons, a tiny freshwater teleost, the medaka, is an excellent experimental animal (Yamamoto, 1975). In particular, the see-through medaka, which is genetically deficient in pigments, has recently been established. The internal organs of this stock can be seen through the transparent body wall of the living animal. It is recommended as a model for morphological and molecular investigations on organogenesis in the later stages of fish development (Wakamatsu *et al.*, 2001).

Only one report (Iwamatsu, 1994) has addressed details of morphological changes during growth in the medaka, although there have been many reports on the embryonic development of this fish (Yamamoto, 1975; Iwamatsu, 1993, 1997). The transparent see-through medaka

may provide more detailed information on organogenesis during the growth period in the living fish. In order to accumulate biological data on growth of the medaka fish, we tried to observe carefully the sequence of morphological changes during growth of the fish. On the basis of these observations, an aim of the present paper is to establish a standard table that is convenient for determining growth stages from hatching to adulthood in the medaka. The established table of the growth stages is expected to facilitate further experimental investigations to settle the unanswered questions in fish growth underlying morphogenesis.

MATERIALS AND METHODS

The see-through medaka of *Oryzias latipes* (Wakamatsu *et al.*, 2001) were obtained from the Laboratory of Freshwater Fish Stocks, Bioscience Center, Nagoya University (Nagoya, Japan). The living see-through medaka were kept in several 2-liter transparent plastic tanks with a water circulating system (MH: Meito-suisan, Nagoya) at 26°C and under a 14 hr light and 10 hr dark cycle. Adult fish were fed a few times each day on a mixture (1:1) of parched barley flour and shrimp powder or Tetramin (West Germany), and fry were fed on Otohime β 1 (<50 μm grain powder: Nisshin Feed,

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Tokyo). Some fish were fed carbon particles blended with powered Tetramin for more than 1 hr prior to observations of the gut.

In order to compare the development of this strain with another strain, the d-rR strain obtained from the Laboratory of Freshwater Fish Stocks, Bioscience Center, Nagoya University (Nagoya, Japan) was used in some cases.

For observations, fish were anesthetized by placing them in a 90 mm Petri dish with saline (Iwamatsu, 1974) containing about 0.05% phenylurethane. This solution was prepared by adding a mixture of saturated (approx. 0.1%) phenylurethane (7 parts) and ethanol (3 parts). The sizes of internal and external organs and the body were measured in anesthetized fish by using a stereoscopic microscope ($\times 20$, Olympus SZX12) equipped with a calibrated ocular micrometer. Observations were performed at room temperature.

The female was recognized by the gonad (ovary with oocytes) and male by its thread-like thin gonad consisting of a long cell mass.

RESULTS AND DISCUSSION

Growth rate is affected by environmental factors such as temperature, oxygen content, salinity, water stream, population density and food availability. Therefore, growth stages are difficult to determine by times and days after hatching unless the environmental factors are completely controlled during the growth period. In addition to the variation caused by a variety of environmental factors, genetic differences have been known to be expressed early in zebrafish life, even during embryonic development within a brood (Kimmel *et al.*, 1995). For this reason, Sire *et al.* (1997) pointed out that it is better to refer to a developmental table based on precise characters, rather than to age. On the other hand, in the anchovy, events in organ development have been expressed in terms of standard length (O'Connell, 1981). Thus, in the present study, the growth stages were represented by several distinct morphological characteristics and total length (TL; the distance from the anterior tip of the lower oral jaw to the posterior edge of the caudal fin).

In fish (about 4.5 mm TL) that had just hatched, rudiments of most organs formed in the embryonic period could already be recognized, except for some external ones such as fins and scales. All the growth stages are illustrated diagrammatically (Fig. 1).

Blood vessels: At the time of hatching, the large dorsal artery below the notochord still remained separated about 0.3 mm from the ventral vein which ran along the ventral edge of the tail (Fig. 1). The ventral vein turned downward towards the ventral edge of the tail at the 28–29th vertebra. In fish 6.0–7.5 mm TL, the vein was fragmentarily formed in contact with the artery. When TL reached 7.8 mm or more, the two blood vessels began to lie in continuous contact with one another (parallel vascularization) within the hemal foramen of hematospines beneath the vertebrae in the posterior vertebral (the 13–30th vertebra) region of the tail, although the blood vessels still separated in the 8–12th vertebrae by a distance of 0.18–0.24 mm. In fish more than 8.0 mm TL, the ventral vein became poor and degenerated to a very minor vein.

Fins: In the newly hatched larvae, both the dorsal and anal transparent fin folds were continuous to the caudal fin folds, but the ventral fin fold was separated in front of the anus. In larvae 4.5–4.9 mm TL, fin rays began to form earlier on hypurals in the unpaired caudal (4–5 rays) and the paired pectoral fin folds (4–5 rays). The oil droplet disappeared coincidentally with absorption of the yolk mass, just before appearance of nodes (joints) in the caudal fin rays when the fish grew to approximately 5.5–6 mm TL. The mean number of ray nodes in the caudal fin increased in proportion to the increase in TL (Fig. 2). In fish 6.7–7.0 mm TL, the rays in the anal fin and the nodes in the pectoral fin rays formed (Fig. 1). This is just before the unpaired anal and the dorsal fin folds were dramatically transformed to the adult fin shape by the restricted degeneration of membranous fin folds in front of the caudal fin. The mean number of ray nodes in the pectoral fin also increased linearly during growth (Fig. 2).

The rays in the dorsal fin and the ray nodes in the anal fin first appeared when TL reached 8 mm. In the fish of this size, the pectoral fins already had the same number of rays (9–10 rays; Fig. 3) as in the adult (8–11, Iwamatsu *et al.*, 1984; 9–11, Iwamatsu, 1986). A pair of bud-like ventral (pelvic) fins newly formed on both lateral sides separated somewhat from the ventral membranous fin fold (Fig. 1) at about 9 mm TL just before the ray nodes appeared in the dorsal fins of fish 10.8–11.0 mm TL (Fig. 2). In fish about 11 mm TL, in which the rays in the paired ventral fins first appeared, the ray numbers of the anal (17–18 rays) and caudal (20 rays) fins equaled those of the adult (17–20 rays in the anal and 18–22 rays in the caudal, Iwamatsu and Hirata, 1980; Iwamatsu, 1986; 17–22 rays in the anal and 17–23 rays in the caudal, Iwamatsu *et al.*, 1984) (Fig. 3). In the ventral fins, ray nodes (2–5 rays) appeared concomitantly with establishment of the ray number (5–7; Iwamatsu and Hirata, 1980; Iwamatsu *et al.*, 1984; Iwamatsu, 1986) when TL reached about 12.4 mm (Fig. 3). The ventral fin fold had disappeared in fish about 12.5 mm TL. The ray number (5–6 rays) in dorsal fins of the adult (5–6, Iwamatsu, 1986; 5–7, Iwamatsu and Hirata, 1980; 6–7, Iwamatsu *et al.*, 1984) was established in fish 13.8–15.8 mm TL (Fig. 3).

A single dichotomous branching in the tip of the fin ray (1–6 rays) first formed with elongation of the caudal fin in fish about 16 mm TL (Table 1). The fin rays were also successively branched in the pectoral, ventral and dorsal fins of fish 17.4–18.3 mm TL. In fish 25–26.5 mm TL, formation of the second dichotomous branching was more advanced in the rays of the caudal, dorsal and pectoral fins than in the anal and ventral fin rays of fish 30 mm TL.

Scales: Scales were not recognizable on fish less than 8 mm TL. The first round scales appeared on the mid-lateral lines of the body in fish 9–10 mm TL (Fig. 1). This indicates that there is individual variation in the time the first scale appears in the see-through medaka strain. Similar individual variation in scale development has been reported in *Rivulus marmoratus*, suggesting that scale development is correlated with both the size and age of fish (Park and Lee,

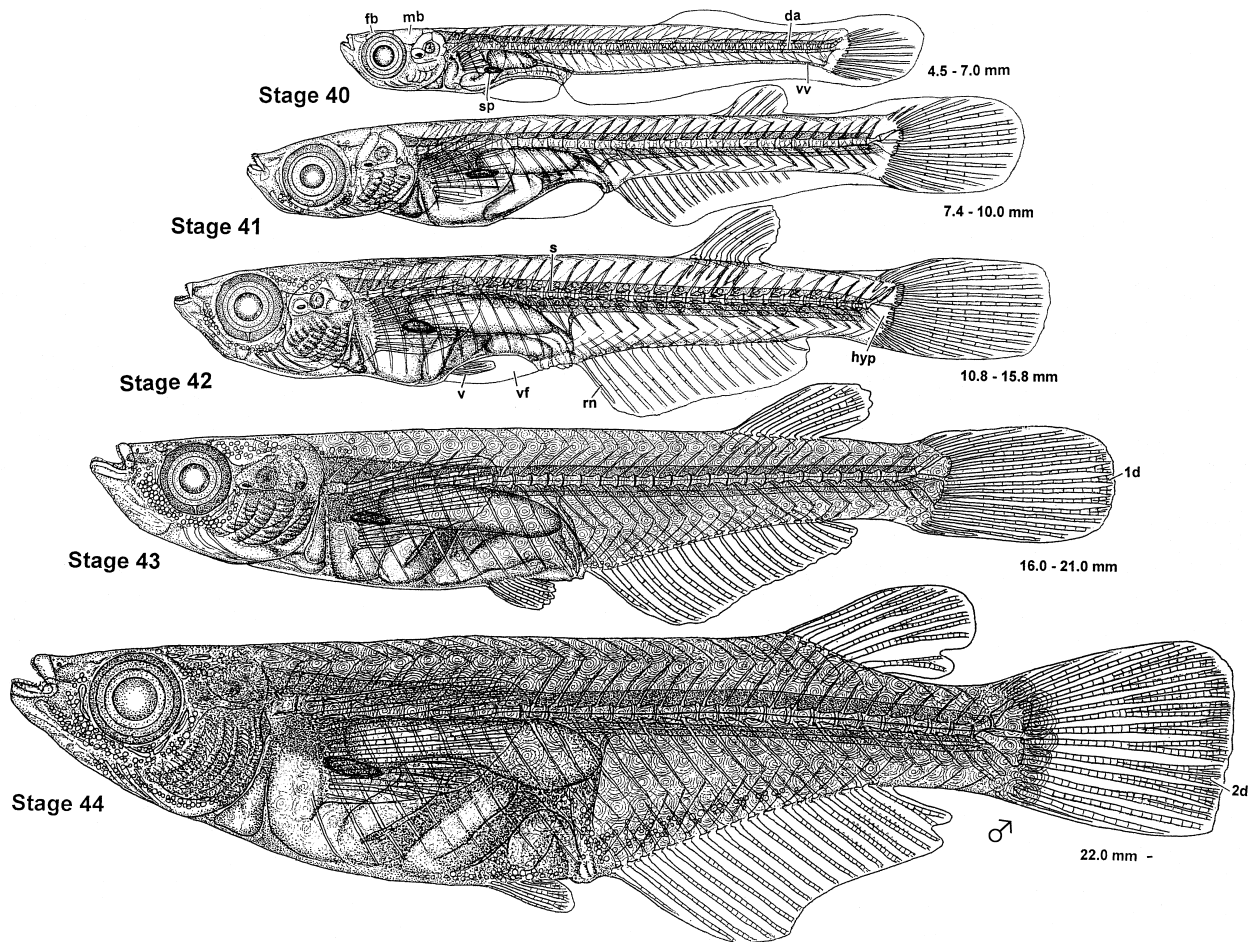


Fig. 1. Diagrammatic illustration of the growth stages of the see-through medaka. Stage 40: the initiation of the fin ray formation (4.5–7.0 mm TL). Stage 41: formation of the fin shape (7.4–10.0 mm TL). Stage 42: establishment of fin ray number (10.8–15.8 mm TL). Stage 43: formation of the first dichotomous branching in fin rays (16.0–21.0 mm TL). Stage 44: formation of the second dichotomous branching in fin rays (more than 22.0 mm TL). 1d or 2d, single or double dichotomous branchings; da, dorsal artery; fb, forebrain; hyp, hypural; mb, midbrain; rn, ray node; s, scale; sp, spleen; v, ventral fin; vf, ventral fin fold; vv, ventral vein.

1988).

The progression of scale formation from the posterior region towards the anterior region of the body was not clear in this fish, unlike *Poecilia reticulata* (Sire and Arnulf, 1990) and *Danio rerio* (Sire *et al.*, 1997) in which the scales form progressively from the posterior to the anterior region of the body. The appearance of scales progressed from the mid-lateral line to the dorsal and ventral edges of the body, and scales were distributed on the whole body surface by 16 mm TL. The number of scales along the midlateral line in adult fish was first counted as 28–32 (*O. latipes*, Iwamatsu *et al.*, 1984) or 30–32 (Iwamatsu and Hirata, 1980; Iwamatsu, 1986) in fish 11 mm TL. In fish about 10 mm TL scales with 4 ridges or more were imbricated with each other. There were more anterior ridges than posterior ridges on scales in the mid-lateral region of the trunk, and the numbers increased in proportion to the total length (Fig. 4). This difference in ridge distribution is probably related to scale imbrication of the exposed posterior region with the anterior region, which is deeply embedded in the dermis.

Gut: Just after hatching, the alimentary tract of the larvae was the embryonic type (Ikeda, 1959) that already curves slightly to the left side of the air bladder (Iwamatsu, 1994). The short oesophagus (foregut) continued to the midgut at the left side of the body. The midgut was larger in diameter than the foregut. The hindgut crossed from the left side to the right anterior region along the ventral side of the body cavity. From this point the slightly narrower hindgut continued spirally to the anus along the dorsal side of body cavity. An open anus was first observed in fish about 5.3 mm TL. In fish about 11 mm TL, the hindgut traveled spirally from the left side towards the posterior right along the dorsal side of body cavity. The shape of the gut also began to transform from a single (~11 mm TL) to a double (~16 mm TL) spiral pattern (Fig. 5). It turned to the left again, then towards the posterior right region along the ventral side, and finally reached the anus from the dorsal side. Thus, the adult (more than 18 mm TL) gut made three spiral turns on the body axis from the foregut to the anus. From a wild *O. (Aplocheilus) latipes* that was collected from Ogikubo in

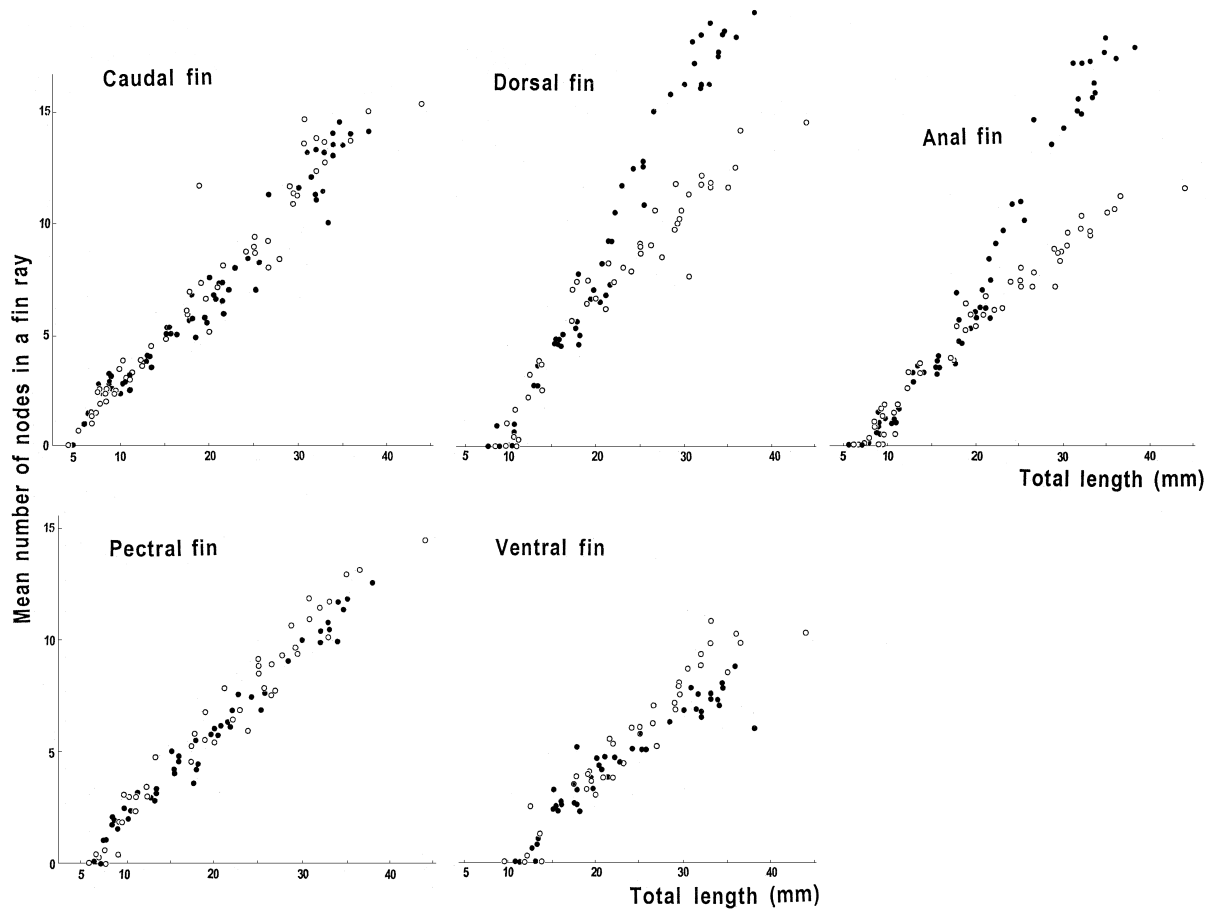


Fig. 2. Change in the mean number of nodes in a fin ray during growth. Open circles indicate the mean number of nodes in female fins and closed circles in male fins.

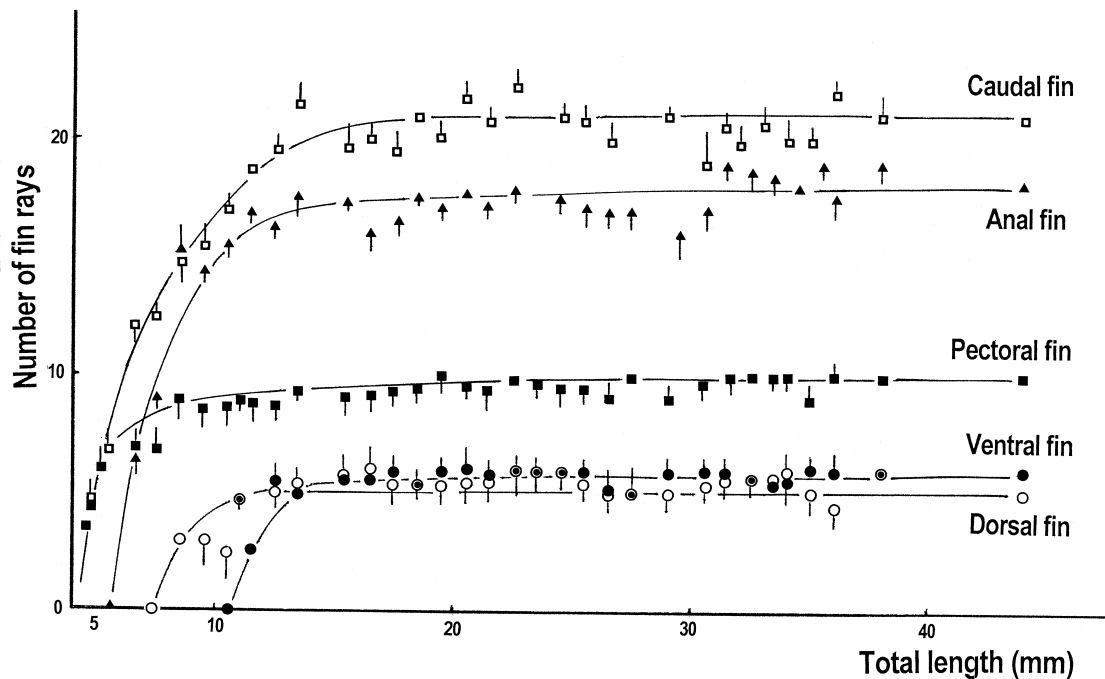
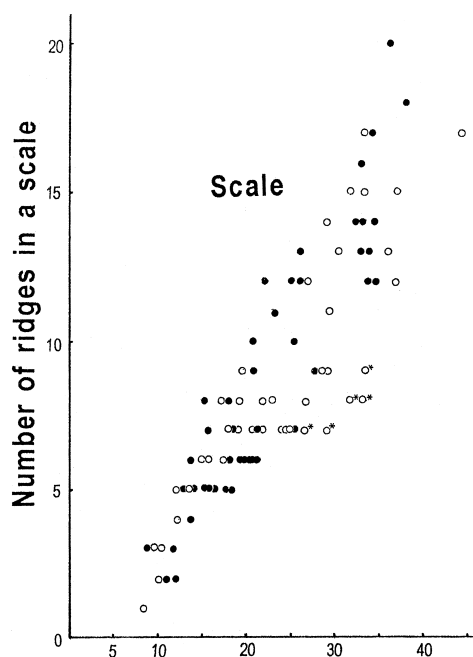


Fig. 3. Change in the number of fin rays in each fin during growth. The vertical bars are standard errors of the mean values.

Table 1. Growth stages of see-through medaka on the basis of morphological features during growth

Total length (mm)	Morphological feature (No. of fin rays)	Stage of growth	Total length (mm)	Morphological feature (No. of fin rays)	Stage of growth
4.5–4.6	C-AR (4–5)		7.4–9.0	PV	
4.8–4.9	P-AR (4–5)		8.0	A,C&D-FF, P-ERN (9)	
5.3	C-AN (4)	40	8.0–8.1	A-AN (1–6), UT	41
5.8	Non-O, ANS		8.0–9.0	AB (gas)	
6.7–6.8	A-AR (6–7)		8.1–8.5	D-AR (3–4)	
6.8–7.0	P-AN (2–3), FR		9.0	V-FF, AO	
			10.0	VC, AS(2)	
10.8–11.0	D-AN (1–5)		16.0–16.2	C-1stDB (2–4)	
11.0	V-AR (3–4), C-ERN (20)		17.4–17.8	UGP (), P-1stDB (1–2)	
11.0	A-ERN (17–18)	42	17.4–19.0	V-1stDB (1–2)	43
11.0–11.1	Gut-2 spirals		18.0–18.3	D-1stDB (1–2)	
12.4	V-AN, V-ERN (5–6)		21.0–21.6	A-1stDB (1–2)	
13.8–15.8	D-ERN(5–6)		21.0–24.4	A-PP(4–7)()	
22.0–25.0	Gut-3 spirals				
25.0–26.0	C-2ndDB (2–6)				
26.5–30.0	P-2ndDB (2–4)	44			
26.5–31.5	P-PP (1–2) ()				
26.5–31.0	D-2ndDB (1–3)				
30.0	A-2ndDB (8)()				
30.0	V-2ndDB (2–3)				

In the anal (A), caudal (C), dorsal (D), pectoral (P) and ventral (V) fins, the first appearance of fin rays (AR), ray nodes (AN) and the established number of fin rays (ERN) is summarized, with formation of single (1stDB) and double (2ndDB) dichotomous blanching at the distal end of fin rays. AB(gas), the first appearance of the air-bladder containing gas; ANS, the first appearance of neural spines on the vertebra in the trunk; AO and AS, the first appearance of the otolith (asteriscus) in the lagena and the scales; FF, formation of the shape of fins; FR, formation of the ribs; Gut-2 and Gut-3 spirals, two and three rotations of the gut; Non-O, absence of oil droplet; PP, papillar processes on fin rays; PV, parallel vascularization of the artery and the vein; UGP, urinogenital protruberance; UT, the first appearance of teeth on the upper jaw; VC, complete vertebral column. The numbers in parentheses indicate numbers of fin rays or ridges on the scale.

**Fig. 4.** Changes in scales during growth. Circles with an asterisk indicate the numbers at the posterior ridge of the scale. See Fig. 2 for symbols.

Tokyo, six rotations (spirals) of the gut were drawn by Suehiro (1942). At present, it is unclear whether or not this difference is related to their habitat locality or strains.

Spleen: The flat, long, reddish spleen was located at the dorsal side of the foregut beneath the anterior left region of the gas-bladder (Fig. 1). The spleen increased in length as fish grew, but varied individually (Fig. 6).

Brain: Widths of both the fore-brain (cerebral) and mid-brain (optic lobe) increased steeply in the early period from hatching to 11 mm TL, after which the rate of increase slightly slowed (Fig. 6). Growth of the brain may correlate with that of the head (Fig. 6), since their growth patterns were very similar.

Vertebra: In fish less than 5 mm TL the vacuolized notochord resembled that of the embryo, and individual vertebrae were difficult to recognize. The dorsal neural spine (neurapophysis) of vertebrae in the trunk already appeared in advance of the hemal spine (haemapophysis) at hatching. When TL reached about 5.5 mm, the early short hematospine first appeared in the tail, and the hemal foramen was still open at the distal end of the hematospine. Vertebrae with both neural and hemal spines were first recognized in fish 5.5 mm TL, and the vertebral bodies (centrum, 0.11 mm in length) were observed between the 1st and 17th vertebrae. A complete vertebral column with well-organized, non-

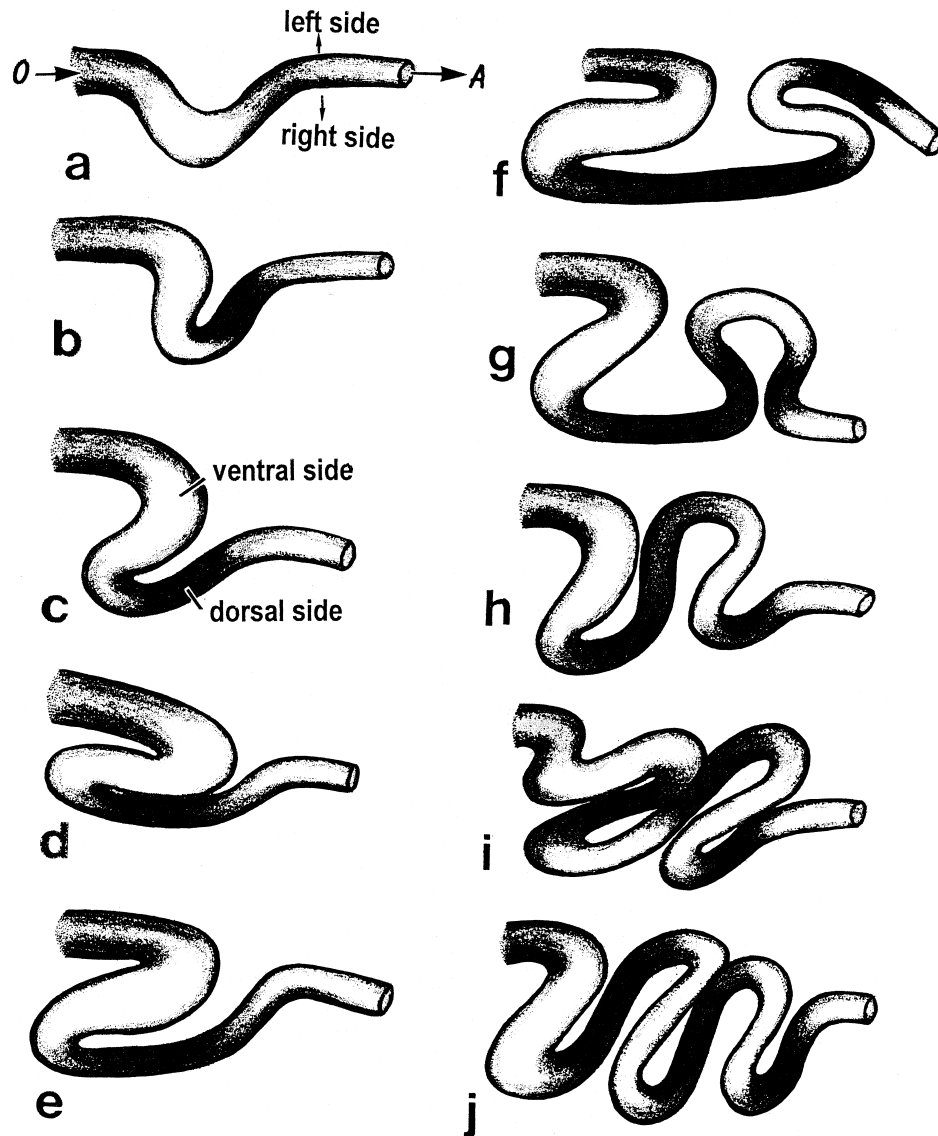


Fig. 5. Change in the shape of the gut during growth. The guts were viewed from the ventral side of the body. Arrow A: the direction toward the anus. Arrow O: the direction from the oesophagus.

vacuolized vertebral body (centrum, about 0.18 mm in length and 0.14 mm in diameter) was observed in fish about 10 mm TL.

The neural spines were flat and wide between the 1st and 10th vertebrae in the trunk, which still did not have ribs. When TL reached 5.7 mm, the snare drum-shaped centrum was about 0.12 mm and 0.10 mm in length at the ends of the trunk and tail, respectively. In fish more than 6 mm long, the hypurals were distinctly visible (Fig. 1). The ribs formed fragmentarily along the boundary of myotomes in the trunk of fish 5–7 mm TL.

Teeth: In larval fish just after hatching, no or a few poor teeth can be seen only on the lower oral jaw. By about 5.2 mm TL, the lower jaw usually had only two teeth. Then, in most cases, the first appearance of teeth on the upper oral jaw were in fish about 8 mm TL.

Otolith: In the inner ear of fish just after hatching, two

otoliths, lapillus (about 60 μ m in diameter) and sagitta (about 80 μ m in diameter), existed already. The otolith (asteriscus) in the legena first appeared in fish more than 9 mm TL.

Sexual differences during growth

Urinogenital protuberance: The earliest sexual difference was recognized as the urinogenital protuberance (UGP) or eminence around the urinogenital pore of females about 17.4 mm TL. The medulla of the UGP appears in response to both estrogen and androgen, but its development is more sensitive to estrogen than to androgen (Yamamoto and Suzuki, 1955).

Fin: There were more ray nodes in the dorsal and the anal fins of adult males were greater in number than in adult females (Egami, 1975). Administration of androgen to females induces development of their dorsal fins. The sex-

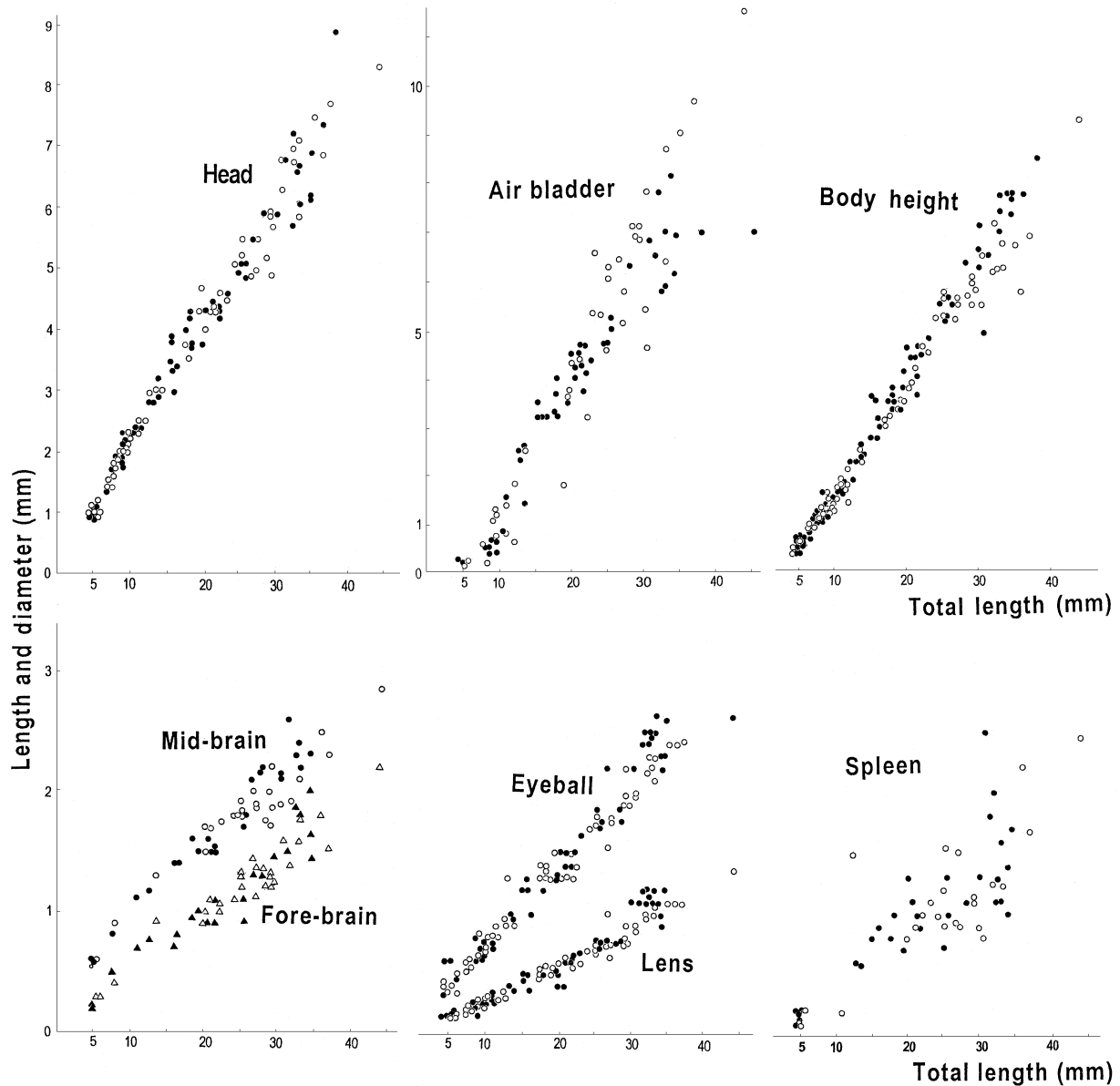


Fig. 6. Change in the length of the air bladder, and the body height, the brain, the head, the spleen and the diameter of the eye during growth. Open and closed triangles indicate females and males, respectively. See Fig. 2 for symbols.

dependent characteristics of the anal (Figs. 2 and 7), dorsal (Figs. 2 and 7) and ventral (Figs. 2 and 7) fins were recognized as differences in the length and in the mean number of ray nodes just before TL reached about 20 mm. The data on the changes in the male anal and dorsal fins during growth coincide with the previous observation (Iwamatsu, 1976). The paired pectoral fins also exhibited sexual dimorphism. The mean number of nodes in a ray of the adult female was definitely greater than in the adult male (Fig. 2)(Egami, 1975). However, there was little sexual difference in the length of the pectoral fins (Fig. 7). This implies that each segment of the pectoral fin rays was shorter in the female than in the male. The papillar processes on the segments of the anal fin rays first appeared in adult males more

than 21 mm TL. In adult males the processes were present on the 2nd to the 8th fin ray counting from the fin ray at the posterior end (Oka, 1931). The adult male had only a dichotomously branched fin ray at the posterior end of the anal fin, while the typical adult female possessed fin rays which were bifurcated dichotomously at each distal end. The dichotomous branching of anal fin rays was first recognized in females more than 21 mm TL.

In the male fish, the ventral fin reaches only to the anterior margin of the anus, while the female ventral fins are longer and go beyond the anus to reach the anterior base of the anal fin (Oka, 1931; Egami, 1956). The present data indicate that the node number and length of female ventral fins increase linearly, but development of male ventral fins

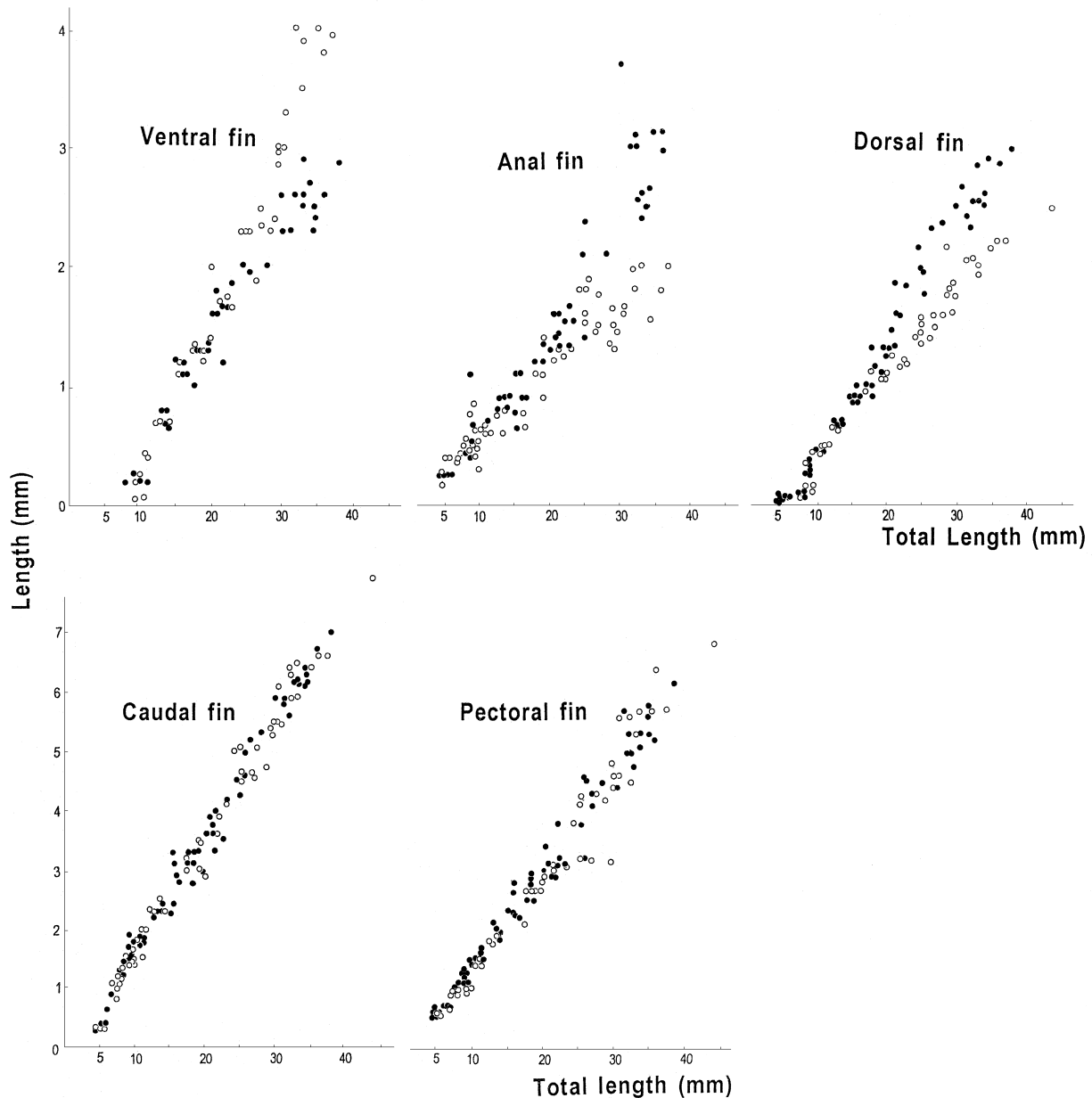


Fig. 7. Change in the length of fins during growth. See Fig. 2 for symbols.

is depressed slightly after sexual maturation (about 20 mm or more TL; Figs. 2 and 7). Suzuki-Niwa (1959) experimentally demonstrated that the shorter ventral fin of the male is a result of the inhibitory effect of androgen. In the pectoral fin of the male, very poor papillar processes also formed on some segments near the distal end of the 2nd fin ray, similar to what has been observed on the rays of the male anal fin (Egami and Ishii, 1956). These poor papillar processes appeared on male pectoral fins at 26.5–31.5 mm TL in a later stage of growth, as compared with those on male anal fins.

The length of the caudal fin did not exhibit a sexual difference and linearly increased in proportion to TL (body length plus the caudal fin length) of the body. In the present

study, the presentation of fish growth by TL instead of standard length (body length) may therefore not be a serious problem.

Body height: The body height was relatively larger in adult males than in adult females (Egami, 1975). This difference was recognizable in fish more than 25 mm TL (Fig. 6).

Air-bladder: Gas was not present in the air bladder before hatching. The air bladder diverticulum containing gas was first observed at 8 mm TL. In most larvae (about 80%) of normal d-rR medaka the air bladder started filling with the gas within 24 hr after hatching. The length of the air bladder in females, but not males, increased linearly, and this increase in length slightly slowed after sexual maturation about 25 mm TL (Fig. 6).

Eye: The mean size of the eyeball increased faster than that of the lens, and these measurements in adult males were significantly larger than those in adult females (Fig. 6).

As summarized in Table 1, the present study proposes that the stages of growth in the see-through medaka may be established on the basis of morphological features. In wild *Oryzias latipes*, there is a variation in the number of fin rays dependent on local groups of fish (Egami and Yoshino, 1958). The present study is the first to observe individual variation in morphological changes in various organs such as the spleen and air bladder during growth of the see-through medaka.

ACKNOWLEDGEMENTS

This study was supported by a grant for the Development of Innovative Technology and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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(Received January 22, 2003 / Accepted February 25, 2003)