Growth of the Medaka (I) – Formation of Vertebrae, Changes in Blood Circulation, and Changes in Digestive Organs

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ABSTRACT

The transient larval structures of vertebrae, blood vessels and the digestive tract were carefully examined during growth. Constrictions appear in the notochords of larvae prior to formation of the vertebral column. Larvae develop the dorso-lateral condyles between the first vertebra and the exoccipitals, differentiated neural and hemal spines, pleural ribs and epipleurals. The digestive organs exhibit sequential development but structures are not lost or degenerated with growth. The ventral vein beneath the cardinal aorta fragments and degenerates during the transition of larvae to juveniles, and is replaced by the dorsal vein. The formation of the dorsal vein completes beneath the cardinal aorta in larvae about 7.0 mm TL. This apparent shift of the ventral to the dorsal vein in the tail differs from the subsequent shift in the trunk where the ventral vein does not degenerate. The dorsal vein completes from the trunk to the tail until 14 mm TL.

INTRODUCTION

The body of fishes has a primitive form and is considered to be the ancestral form for higher vertebrates. Growth of the great variety of forms is exquisitely repeated from generation to generation in different environments. Therefore, their growth provides the opportunity to acquire important fundamental knowledge on the vertebrate body. Comparative anatomy of body structures is an indispensable instrument for the classification of homological aspects. The study of the growth of the fish is not only of great scientific interest in its own right, but also provides phylogenic information on the other vertebrates. The basic biological science of comparing and understanding the intriguing differences between the fishes and the human is especially significant for education at the level of the elementary school (Iwama et al., 2009).

Body size is attained in a relatively short time in short-lived species such as the medaka, a freshwater teleost. The medaka provides an exemplary model for the growth of most teleosts, and may show how the shapes and sizes of the body are determined in life. The wild medaka exhibits a sigmoid pattern of increase in size with age (Iwamatsu, 2002). Major factors involved in the irregular growth or in the limitation of growth may be both genetic and environmental. Environmental factors that determine breeding conditions for fish include temperature, light, food, water, population density and so on. The fish's susceptibility to each of these factors is genetic, species-specific, and also affects growth rate. In the medaka, externally fertilized eggs hatch in water as yolk-sac larvae, which rely on their yolk for nourishment. The yolk sac larvae with their imperfect sensory, circulatory, muscular, and digestive systems can swim freely and feed on plankton. After consumption of the yolk, their body features are still larval. As the fry grow, the morphological forms of the body lose larval characteristics and gradually transform into those of juvenile, subadult (juvenile adult) and finally adult fish. The continuous ontogenetic processes of the morphological transformation between the larval and juvenile body forms are called metamorphosis. During this transitional period of growth, the fish lose specialized larval characteristics and attain subadult characteristics (Kendall et al., 1983).

We have observed growing medaka fish in order to understand the developmental processes and basic patterns of body formation in this lower vertebrate. During their growth period, the stage of development is identified based on several fundamental morphological and morphometric criteria. So far, the transitional process of each feature of the body that defines the growth stage has not observed in detail, although the stages from larva to adult of the medaka were previously described in a previous report

(Iwamatsu et al., 2003). The present paper will provide data regarding the morphological changes in the blood circulation and the digestive tract, as well as vertebral formation in growing medaka during the transformation stages 41–43. Events in organ development are expressed relation to total length.

MATERIALS and METHODS

Medaka fish, *Oryzias latipes* (d-rR strain), used in the present study were reared in a rectangular glass aquarium (60×35×30 cm, about 60 liter of water) under reproductive conditions (L14 D10, 26–28°C). Under these conditions, females spawn as they mate with males every day. After spawning and fertilization, the eggs with hardened chorions hang in clusters from the belly of each female. We used our fingers to collect the eggs from the netted females. During the rearing period, the fish were fed a balanced diet containing one part each of shrimp powder, parched barley flour, Tetramin and Otohime no. 1 (Nisshin-seifun).

For observations, live fish were anesthetized in saline containing a mixture of 7 parts of phenylurethane and 3 parts of ethanol. The sizes of the whole body, internal organs and external organs were measured in anesthetized fish using a stereoscopic microscope (×20, Olympus SZX12) equipped with a calibrated ocular micrometer. Body size is represented by total length (TL, from the snout to the extremity of the caudal fin) by which the stage of fish development is classified, because the rate of growth is perturbed by wide temperature variations.

For examinations of skeleton, deeply anesthetized fish were fixed in 2% glutaraldehyde-saline for one hr. After they were rinsed in distilled water, the specimens were treated for one min in 0.5 N NaOH and stained with 0.5% alizarin red S (phosphate buffered to pH 7.0) for several hours. The alizarin-stained specimens were again treated with 0.5 N NaOH for a few hours, completely rinsed in tap water and finally cleared in 50% glycerol for observation. Growth stages of the fish follow the author's developmental criteria (Iwamatsu, 1994, 2004, Iwamatsu et al., 2003). Names for skeletal structures were traditionally those assigned by Owen (1984) and Yabumoto and Ueno (1984). Abbrevations of anatomical technical terms used in the first paper are given as follows: a, anus; af, anal fin fold; bac, basioccipital; bd, bile duct; c, centrum; con, constriction; dt, digestive tract; ep, epipleural; gb, gall bladder; hs, hemal spine; nc, notochord; ns, neural spine; pd, pancreatic duct; poz, postzygaphysis; pp, parapophysis; rb, rib; sp, spleen; vf, ventral fin fold; vv, ventral vein.

OBSERVATIONAL RESULTS and DISCUSSION

At hatching the medaka still has a large yolk sphere but begins to feed freely (about 4.5 mm TL). These yolk-larvae already possess most organs in a rudimentary or incomplete state. Kendall et al. (1983) has described the larval stage from hatching to attainment of complete fin ray counts and the beginning of squamation in *Trachurus symmetricus*, providing the most general scheme of terminology for early development of fishes. In the medaka, this larval stage may correspond to the period from stage 40 to stage 42 that establishes complete fin ray number and accompanies the appearance of ray nodes (segments) in all fin rays. These stages are characterized by a loss of specialized larval characteristics and attainment of adult characteristics.

In the medaka, the transformation stage from the larva to the juvenile is characterized by the appearance of the fish as small adults (subadult) and is completed by about 15.8 mm TL. The juvenile fish in stage 43 attains additional adult structures such as dichotomous branching in the tips of the fin rays, increase in scale ridges and the secondary sex characteristics until stage 44 (Iwamatsu et al., 2003), although it looks similar to the adult (Fig. 1).

1) Development of vertebrae and ribs

The sequence of formation and development of ossified structures such as skulls, vertebrae, ribs, fin skeletons and scales has provided excellent developmental characteristics to be used for inductive and systematic inferences. Development of acellularity in the vertebral bone of the medaka has been investigated using histological and fluorescent microscopic analysis by Ekanayake and Hall (1987). They demonstrated that acellularity in the vertebral bone is not due to secondary transformation of an early cellular bone during ontogeny. Osteoblasts withdraw from the site of bone matrix formation, never becoming entrapped in matrix, and the bone matrix is only deposited from the outer surface of the vertebral bone that arises directly as a primary tissue around the notochord. According to these investigators, in embryos at Matsui's stage 28, the notochord appears as a rodlike structure filled with large vacuolated notochord cells and surrounded by mesenchymal cells.

The vacuolation of the notochord begins at embryonic stage 26 (Iwamatsu, 1994, 2004). Embryos at stage 33 do not yet display the constriction in the notochord that marks the junction of a pair of vertebrae in the adult. In embryos at stage 39, one day before hatching, the area between two adjacent constrictions develops into a single vertebra, consisting of a core of notochord

surrounded by a sheet of vertebral bone (Ekanayake and Hall, 1987). The position of the constrictions corresponds to the border between somites.

In larvae at stage 40 (4.5 mm TL) just after hatching, the first vertebra articulates with the skull only by a basioccipital condyle. The dorso-lateral condyles of the first vertebra that articulate to the exoccipitals are formed during the growth period of stage 40 from 7.5 mm TL to 8.5 mm TL, following formation of the median articular bone of the basioccipital. The lateral position of the parapophyses shifts from the upper to the lower sides of the notochord as growth progresses (Iwamatsu et al., 2009 and Fig. 2). In larvae 5.4 mm TL, the upper tips of the neural spines (neuropophysis) in the 1st to 3rd vertebrae are separated from each other, and those of the 2nd – 5th vertebrae attach and form fan-shaped bones which increase from the 1st to the 8th vertebrae (Fig. 2) when TL reaches 10 mm. In stage 41 (7.4–10 mm TL), two projected condyles on the anterior regions of the first vertebra join with two out-growths from the posterior ends of exoccipitals (Iwamatsu et al., 2009, and 7.0 mm TL in Fig. 2).
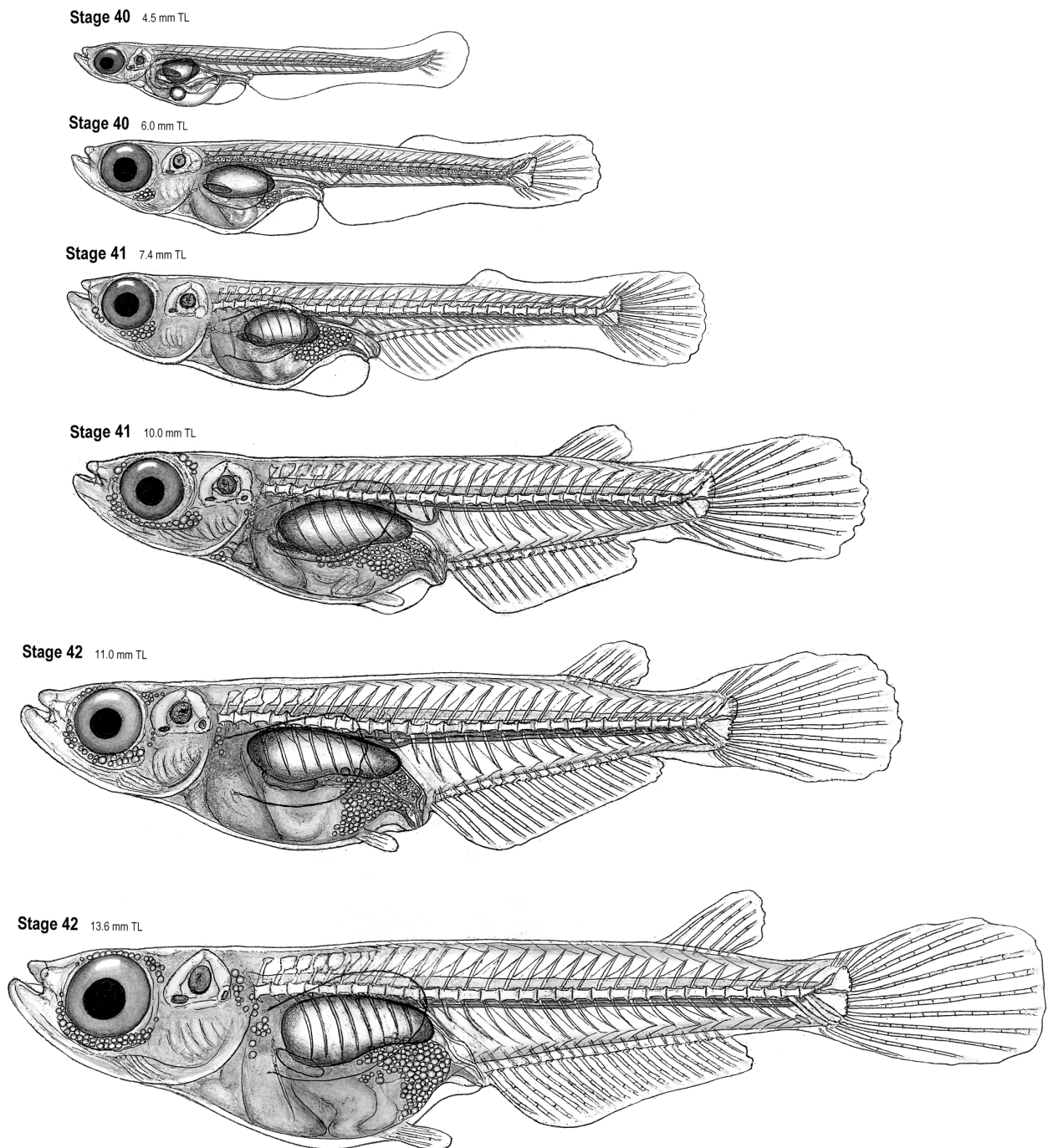

Fig. 1 Diagrammatic illustration of transformation stages in growing medaka.

Stage 40 5.4 mm TL

bac

con

Ventral view

Dorsal view

Stage 40 7.0 mm TL

pp

hs

Ventral view

ns

Dorsal view
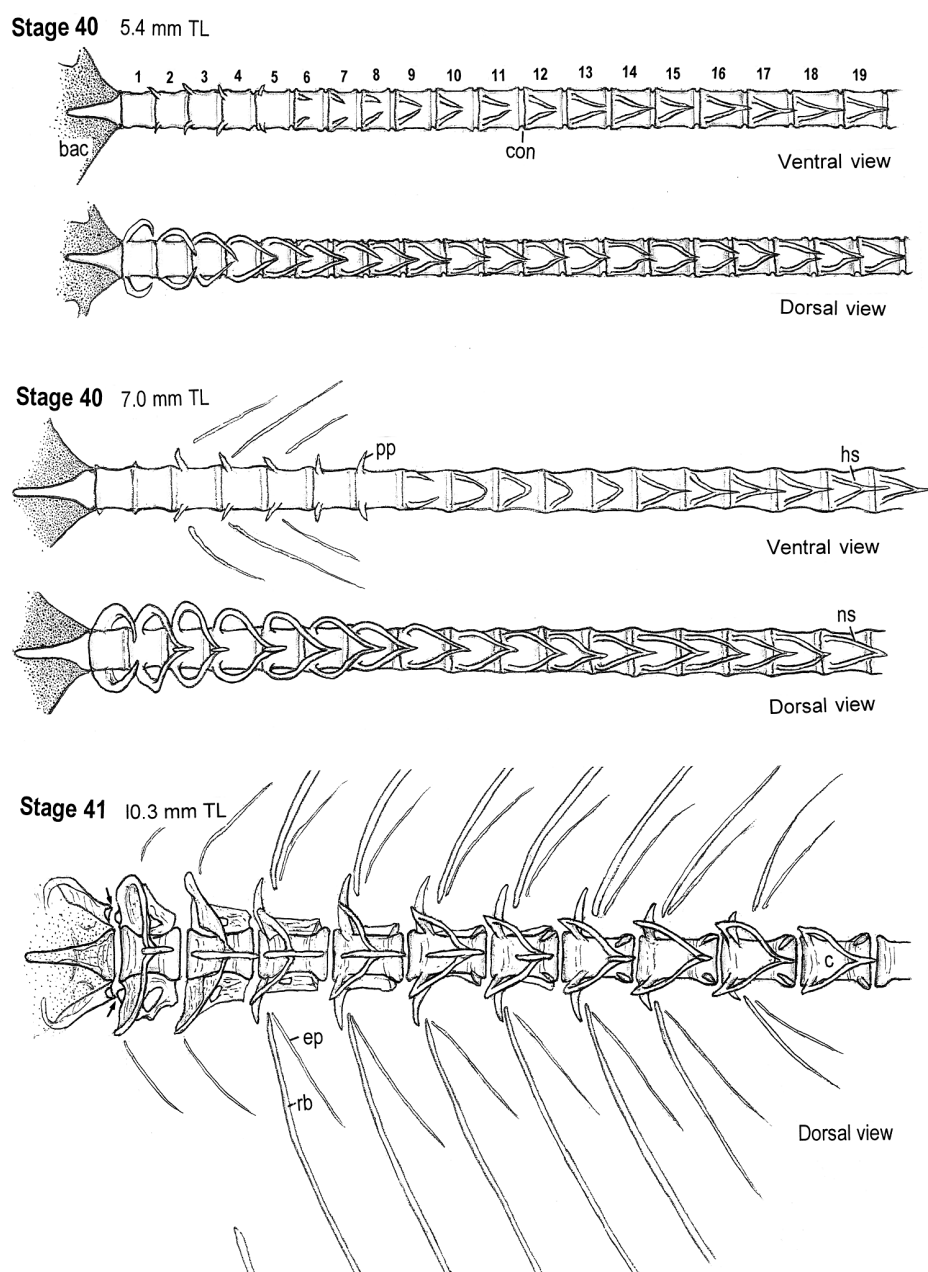
Stage 41 l0.3 mm TL

c

ep

rb

Dorsal view

Fig. 2 Diagrammatic presentation of the anterior region of the vertebrae during growth of the medaka. bac, basioccipital; con, constriction; ep, epipleural; hs, hemal spine; ns, neural spine; pp, parapophysis; rb, rib.

In general, in adults with 30 vertebrae, the centrum of the 1st two vertebrae and a few vertebrae from the caudal end are slightly shorter than the rest (Iwamatsu, 2006; Iwamatsu et al., 2009). In larvae 5.4 mm TL, the transverse processes (parapophyses) occupy the upper-lateral positions of the centrum in the 1st to 5th vertebrae, and in the subsequent vertebrae gradually shift to the ventro-lateral sides. Thus the position of the parapophyses shifts from the upper to the lower regions of the centrum in the posterior part of the trunk as vertebrae proceed toward the posterior region of the trunk (1st – 12th vertebrae). The undeveloped hemal spines (hemapophyses) of caudal vertebrae are not distinguishable from the ventro-lateral transverse processes with their fused distal ends in the 10th – 12th vertebrae in the posterior region of the trunk. At the dorsal side of the notochord, there are neural spines surrounding the neural canals through which the spinal cord runs, although the distal tips of the 1st – 3rd neural spines still remain open. When larvae reach about 5.4 mm TL, the distal tips of all hemal spines come in contact with each other and form the hemal canals in the 14th – 30th caudal hemal spines. The 13th hemal spines at the most anterior vertebra of the tail retain open distal tips (Fig. 2). In larvae more than 7 mm TL, the hemal spines elongate in all caudal vertebrae. In the trunk, the
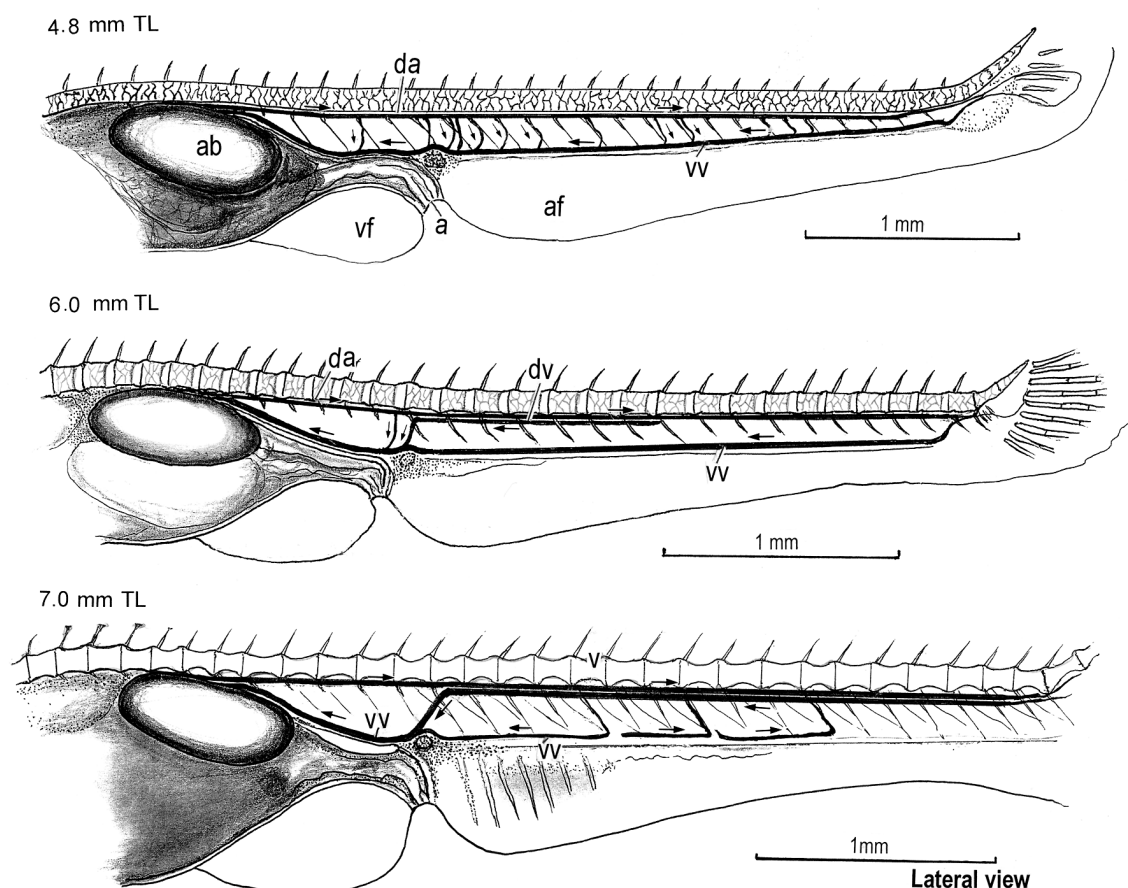
Fig. 3 Shifting off the ventral vein to the position beneath the cardinal aorta in the tail of the medaka.
Arrows indicate the direction of blood flow. a, anus; ab, air bladder; af, anal fin fold; da, dorsal aorta; dv, dorsal vein; vf, ventral fin fold; vv, ventral vein.

distal ends of the 1–8th parapophyses remain separated in larvae 5–7 mm TL, while those of the 9th – 12th parapophyses fuse with each other, so that hemal canals are formed. The scanty pleural ribs appear first between myotomes from the 3 rd to 5 th vertebrae in the trunk, but do not closely attach to the parapophyses. The intermuscular bones or epipleurals are not recognized yet. As seen in Fig. 1, the larva at about 10 mm TL possesses a complete vertebral column with a non-vacuolated centrum (Iwamatsu et al., 2003). Post-zygapophyses are recognized on the dorsal sides of the centrum. In larvae 10.4 mm TL of which the 2nd-9th vertebrae have pleural ribs, the distal ends of all parapophyses are open in the trunk. There are pleural ribs in the 2nd-11th vertebrae of larvae 12–14.5 mm TL. In larvae more than 15.4 mm TL, formation of eleven ribs is completed together with the epipleurals on parapophyses of the 2nd – 12th vertebrae. The vertebral constrictions are first recognized in larvae 5.0 mm TL. The transparent region of constrictions in each vertebral column is observed in larvae 5.4 mm TL.

2) Changes in blood circulation
    In the medaka embryo, the formation of blood vessels begins in embryonic stage 23 (Iwamatsu, 1994, 2004). The formation of the caudal vein is first seen in stage 25. The dorsal aorta develops in the ventral side of the hypochord within the embryonic body. The investigation by Fujita et al. (2006) has provided a detailed characterization of the vascular anatomy of developing medaka embryos. In the embryonic development of the zebrafish, the major axial vessels such as the dorsal aorta and the vena cava are thought to be formed by migration of angioblasts to the site of vessel formation (cf. Eriksson and Lofberg, 2000). However, it has not been ascertained whether the cardinal vein in the medaka tail is formed by angioblasts in a manner similar to that of the dorsal aorta of the zebrafish embryo.
    In stage 40 larvae just after hatching, the main caudal aorta beneath the notochord separates from the cardinal vein which is located in the ventral margin of the tail (Figs. 1 and 3). The ventral vein approaches the dorsal aorta at the 6 – 7th vertebrae
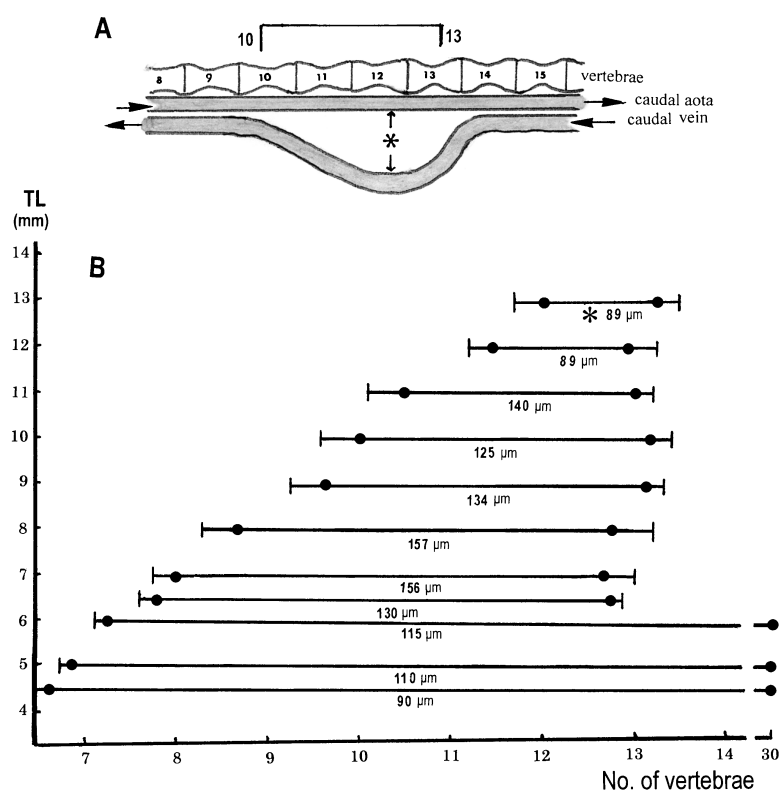
Fig. 4 Shifting of the ventral vein to the position beneath the cardinal aorta in the posterior trunk (anterior parts from the 12th vertebra) of the medaka.
A. Illustration of a positional relationship between separated blood vessels and vertebrae. The figure illustrates separation of the ventral vein from cardinal aorta at the distance of an asterisk (*). B. Horizontal bars with error bars indicate the separated range of ventral vein between vertebrae (black spots). Numbers under each line are distances between separated blood vessels.

in the trunk and further runs parallel to the dorsal aorta along the dorsal side of the air bladder toward the most anterior region of the trunk. Until 5.0 mm TL, small blood streams can be seen leading from the dorsal aorta to the ventral vein along each hemal spine, and especially major blood streams at the 12th – 13th vertebrae. The earliest appearance of a new dorsal vein parallel to the dorsal aorta through the hemal canals is observed between the 20th and 21th vertebrae in the larvae 5.3 mm TL. In larvae 5.8 mm TL, the dorsal vein beneath the dorsal aorta is recognized in the hemal canals between the 13th – 16th, 18th – 20th and 21th – 23th vertebrae. In larvae 6.0 mm TL, the fragmentary dorsal veins are also seen beneath the dorsal aorta in the hemal canals between the 12th – 17th, 8th – 20th and 21th – 23th vertebrae. When larvae grow up to 6.6–7.0 mm TL, an elongated dorsal vein is in the hemal canals between the 13th – 26th vertebrae. During this short period of shifting by the vein, the ventral vein remains scanty in the marginal region of the ventral side of the tail. In the tails of larvae around 7.0 mm TL, the completed dorsal vein is observable and the scanty

ventral vein fragments are still recognizable. Consequently, the dorsal vein parallel to the dorsal aorta is formed in fragments in the hemal canals of the tail during the growth period from 5.3 to 7.0 mm TL (Fig. 3), while the original ventral vein fragments and degenerates. Thus the dorsal vein does not form by shifting of the ventral vein to a new position, as evidenced by the absence of an intermediate for the intact ventral vein from the ventral margin into the hemal canals that are formed by fusion of the distal ends of the hemal spines in developmental stage 41, as described above. Thus, in the tail, the new dorsal vein is formed in fragments through the hemal canals and subsequently the ventral vein becomes meager and degenerates. In the 13th – 30th vertebrae of the tail, formation of the dorsal vein is completed beneath the cardinal aorta.

On the other hand, in larvae more than 7.5 mm TL, the dorsal aorta and the ventral vein remain separated in the posterior trunk between the 8th – 13th vertebrae (Fig. 4). The separation of the two vessels gradually decreases by approach of the ventral vein to the position of the dorsal aorta from the anterior to posterior vertebrae as growth progresses (Fig. 4). Unlike the case of the caudal ventral vein, a long intact ventral vein in the posterior trunk shifts from the ventral side between the open distal tips of the parapophyses, through the vertical septum (median border) that separates the muscles into the left and right halves. However, it is unclear whether or not the ventral vein is shortened or shrinks during shifting. Finally the dorsal vein completes its contact with the dorsal aorta at 14 mm TL (Fig. 1).

3) Changes in the digestive system

To acquire basic knowledge of the digestive system in the medaka, its development should be undertaken using key molecular markers in combination with the morphological pattern of respective mutants.

The medaka hatches with the gut on a reduced yolk mass. Just after hatching, the rudimentary gut of the larvae is of the embryonic type (Ikeda, 1959) that already curves slightly to the left side of the air bladder (Iwamatsu, 1994). The extremely short esophagus (210–300μ and 500–700μ in length, 10–20 mm TL and 25–35 mm TL, respectively) empties directly into the digestive tract through the melanotic diaphragm (front wall of the body cavity). The digestive tract is first located in the left side
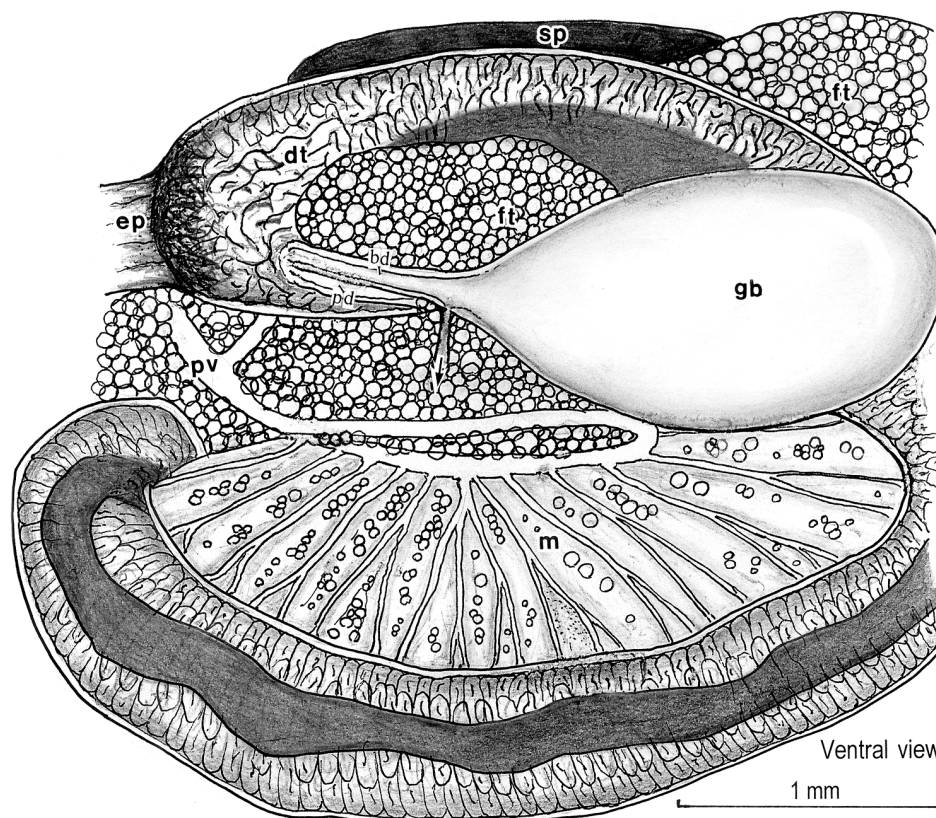
Fig. 5   The anterior area of the digestive tract (dt) showing an attaching point of the bile (bd) and pancreatic (pd) ducts, the portal vein (pv) and the mesentery (m) of the medaka 19 mm TL.
A small arrow indicates the direction of the liver. ep, esophagus; gb, gall bladder; sp, spleen.

of the body cavity and then curves from the left side to the antero-right region along the ventral wall of the body cavity. From the mid-gut, the slightly narrower hindgut continues spirally toward the anus along the dorsal side of body cavity. The medaka lacks a stomach. The bile and pancreatic ducts directly connect slightly to the right side of the anterior margin or anterior portion of the digestive tract (Fig. 5). In the digesive tract, differentiation of the stomach is not observed in the larval stage. The anterior portion of the digestive tract seems to correspond to the duodenum into which the bile and pancreatic ducts open at a distance of about 300–800μ, respectively, from the anterior tip and slightly on the right side of the digestive tract in juveniles 10–25 mm TL (Fig. 6). In some individuals, the bile and pancreatic ducts open at the anterior margin of the digestive tract. The diameters of the gall bladder and bile duct increase with growth (Fig. 6). The diameter of the forepart of the digestive tract increases in proportion to the increase in TL. It is almost twice as large as that of the posterior tract (Fig. 7). The length of the digestive tract is shorter than the TL until about 20 mm TL. However, after this it lengthens faster than the TL (Fig. 8). In the mesentery, the small blood vessels extending from the portal vein become conspicuous (Fig. 5). Subsequently, the digestive tract elongates and is conspicuously coiled depending on development of the mesentery as growth proceeds. In larvae of about 15 mm TL, the length of the digestive tract increases as compared with that of the major blood vessels of the viscera, so that the digestive tract makes a twist and is coiled or folded in the body cavity and finally opens into the anus. The morphological pattern of the adult digestive tract may transform depending on the development of the portal vein, blood vessels in the mesentery and fat tissue in addition to the elongation of the gut itself. Thus, the digestive organs exhibit gradual changes and become the adult type during the transformation period from the larva to the juvenile.

    In short, as described above, the present observational results have pointed out that the medaka exhibits degeneration and attainment of transient structures in vertebrae and blood circulation during the growth period from stage 40 to stage 42 (Iwamatsu et al., 2003).
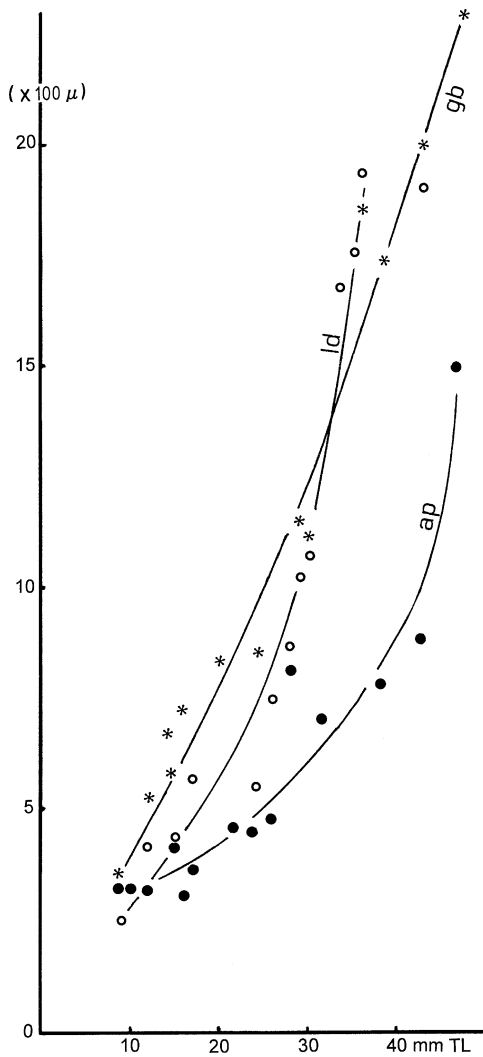
Fig. 6 Changes in the length of the bile ducts (ld), diameter of gall bladders (gb) and the distance of the attaching point (ap) of bile and pancreatic ducts from the most anterior margin of the digestive tract during growth of the medaka.
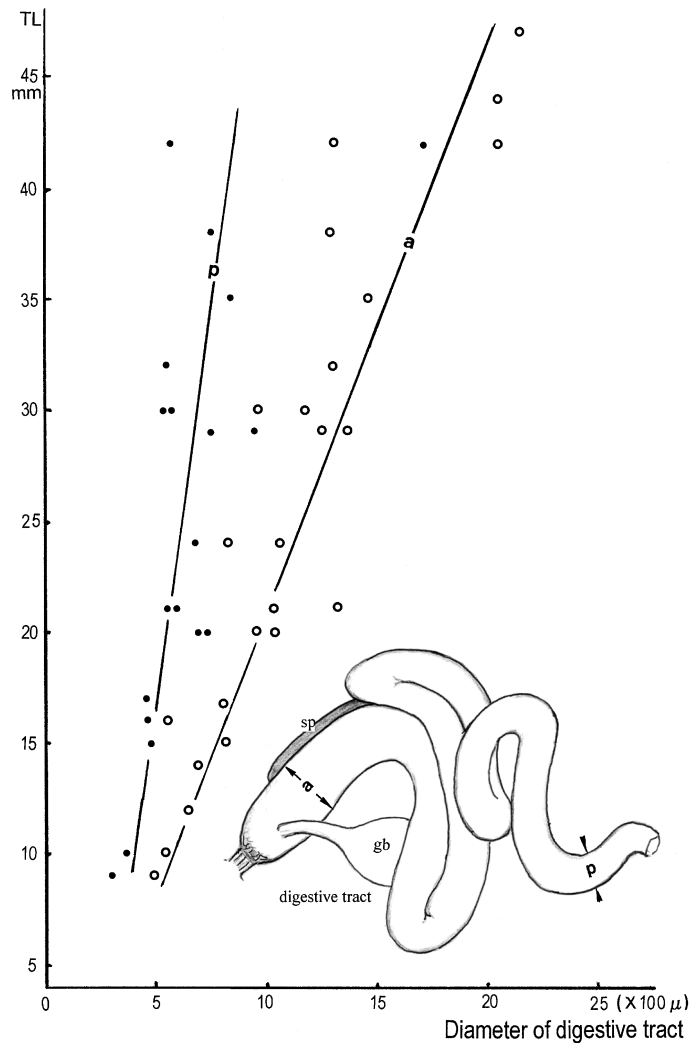
Fig. 7 Changes in the diameter at the anterior (a) and posterior (p) regions of the digestive tract during growth of the medaka.

REFERENCES

Bon, E.B., 1979. Biology of fishes. pp. 401–405, W.B. Saunders Company, Philadelphia.

Brown, D.D.,1997. Proc. Natl. Acad. Sci. USA, 94: 13011–13016.

Egami, N., 1957. Annot. Zool. Japon., 30 (1): 26–30.

Ekanayake, S. and B.K. Hall, 1987. J. Morph., 193: 253–261.

Eriksson, J. and J. Lofberg, 2000. J. Morphol., 244: 167–176.

Fujita, M.,S. Isogai and A. Kudo , 2006. Dev. Dynam., 235: 734–746.

Fukuhara, O., 1987. Nippon Suisan Gakkaishi, 53 (3): 371–379.

Fukuhara, O. and T. Fushimi, 1986. Bull. Jap. Soc. Fish., 52 (1): 75–80.

Ikeda, A., 1959. J. Med. Sci., 8: 71–89.

Iwama, J., S. Matsubara, A. Fukuchi and T. Shimojo, 2009. Kagaku kyouiku kenkyu, 33: 73–85. (In Japanese with English Abstract)

Iwamatsu, T., 1994. Zool. Sci., 11: 825–839.

Iwamatsu, T., 2002. Animate, No. 3, 45–46.
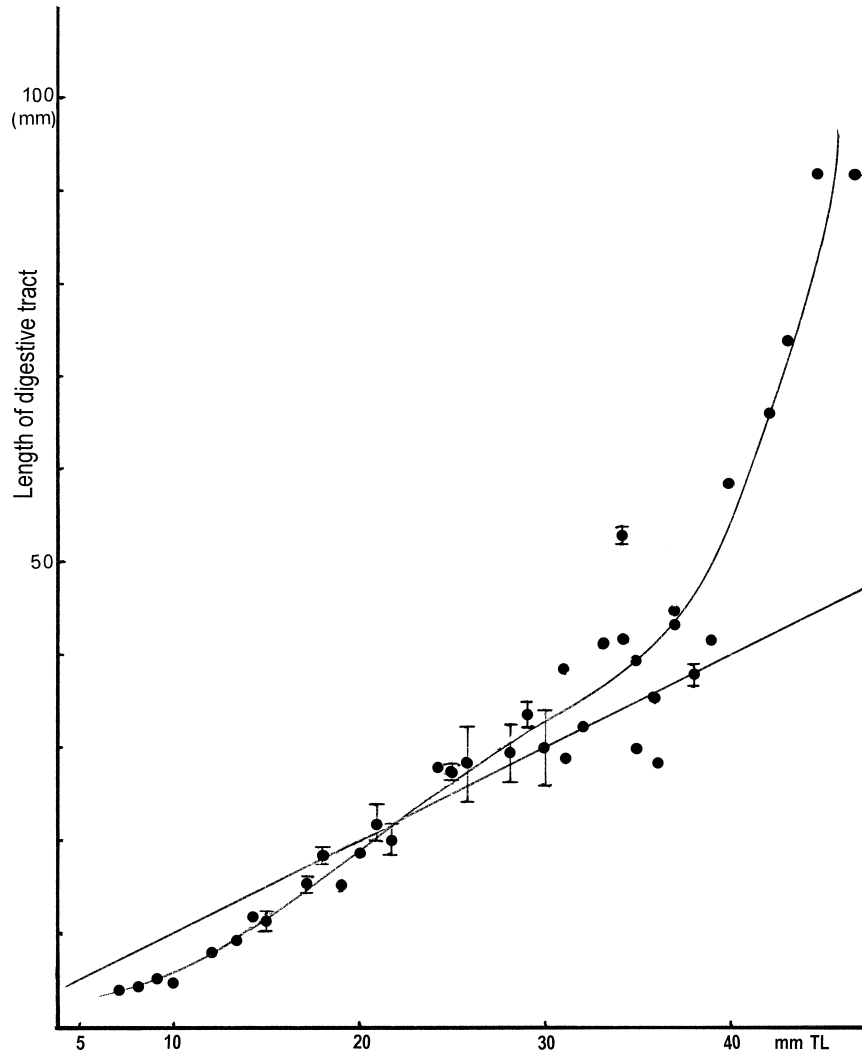
Iwamatsu, T., 2004. Mech. Develop., 121: 605–618.

Fig. 8   Relationship between the length of the digestive tract and total length.
A straight line indicates a correlation between the two lengths.

Iwamatsu, T., 2006. The Integrated Book for the Biology of the Medaka. Daigaku-kyouiku Publ. Co., Okayama, pp. 473. (In Japanese)

Iwamatsu, T., H. Nakamura, K. Ozato and Y. Wakamatsu, 2003. Zool. Sci., 20: 607–615.

Iwamatsu, T., M. Sato and K. Nakane, 2009. Bull. Aichi Univ. Educat., 58: 69–79.

Kendall Jr., A.W., E.H. Ahlstrom and H.G. Moser, 1983. *In*: Ontogeny and Systematics of Fishes – Based on An International Symposium Dedicated to the Memory of Elbert Halvor Ahlstrom. Special Publ. No. 1, Amer. Soc. Ichthyol. Herpet., pp. 11–22.

O'Connell, C.P., 1981. Amer. Zool., 21: 429–446.

Owen, R., 1984. Anatomy of Vertebrates. Fish and Reptiles. International Books and Periodical Supply Service, New Delhi.

Sire, J.-Y., F. Allizard, O. Babiar, J. Bourguignon and A. Quilhac, 1997. J. Anat.,190: 545–561.

Sire, J.-Y. and I. Arnulf, 1990. Jap. J. Ichthyol., 37(2): 133–143.

Tagawa, M. and T. Hirano, 1991. J. Exp. Zool., 257: 360–366.

Tomita, H., 1961. Annot. Zool. Japon., 34(2): 80–85.

Yabumoto, Y. and T. Ueno, 1984. Bull. Kitakyushu Mus. Nat. Hist., 5: 143–161.