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Review

Stages of normal development in the medaka *Oryzias latipes*☆

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Abstract

Unfertilized eggs of *Oryzias latipes* were artificially inseminated and incubated at 26 ± 1 °C. Careful observation of the process of embryonic development by light microscopy allowed division of the process into 39 stages based on diagnostic features of the developing embryos. The principal diagnostic features are the number and size of blastomeres, form of the blastoderm, extent of epiboly, development of the central nervous system, number and form of somites, optic and otic development, development of the notochord, heart development, blood circulation, the size and movement of the body, development of the tail, membranous fin (fin fold) development, and development of such viscera as the liver, gallbladder, gut tube, spleen and swim (air) bladder. After hatching, development of the larvae (fry) and young can be divided into six stages based on such diagnostic features as the fins, scales and secondary sexual characteristics.

Keywords: Medaka; Embryo; Phenotype; Morphology; Developmental stage; Growth stage

1. Introduction

The genus *Oryzias* (the medaka), which inhabits fresh and brackish waters from India throughout South-east Asia across Wallace's line to Timor, Sulawesi, Luzon, and Japan, is unique among common laboratory teleosts. The natural breeding season of *Oryzias latipes* extends from mid-April to late September in Japan. Oocyte maturation occurs at night (Iwamatsu, 1965, 1974), and ovulation at dawn (Egami, 1954; Iwamatsu, 1978). Under regular daily photoperiod with more than 13 h of artificial lighting (Yoshioka, 1963), ovulation occurs about 1 h before the onset of the light period, and oviposition occurs for 1 h before and after the onset of the light period throughout the year. Recently, it has been shown that oocytes can be induced to mature and to ovulate in vitro without any exogenous hormone if they are removed from the ovary less than 12 h before the onset of the light period and incubated in culture medium (Iwamatsu, 1978). The eggs develop to the hatching stage within 10 days at 26 °C. Embryonic development in *Oryzias javanicus* (Iwamatsu and Hirata, 1984) and *O. latipes* follows the typical teleostean pattern; therefore, these transparent eggs are excellent and very convenient materials for investigating fish embryogenesis.

The normal developmental process of *O. latipes* has been reported in Japanese by several investigators (Gamo and Terajima, 1963; Hiraki and Iwamatsu, 1979; Iwamatsu, 1976; Kamito, 1928; Kirchen and West, 1976; Kubo, 1935; Matui, 1949) and in English by Kirchen and West (1976) and Yamamoto (1975). In these reports, the descriptions of Stage 1 to Stage 27 are in good agreement. However, except for our reports (Hiraki and Iwamatsu, 1979; Iwamatsu, 1976) the descriptions of later stages (St. 28 to St. 33–36) of development are rough, sketchy and not based on detailed observations of the development of tissues and organs. The present author presented in Japanese more detailed descriptions of the developmental process of *O. latipes* in 'The Biology of the Medaka' (Iwamatsu, 1993). The definitions of the developmental stages of *O. latipes* provide a foundation for studying the embryology of this fish. The purpose of the present report is to present the information in English and includes some additional observations on the developmental course in reference to the data of the early investigators.

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2. Observational results and descriptions

2.1. Stage 0. Unfertilized eggs

The mature unfertilized egg is an oblate spheroid measuring on an average 1245.9 ± 3.9 μm ($n = 122$) in horizontal diameter and a little less (average 1169.9 ± 4.0 μm, $n = 122$) in vertical diameter. The egg proper is closely surrounded by a thick egg envelope, the chorion. The perivitelline space between the chorion and the vitellus is very difficult to recognize using a light microscope. The micropyle located in the chorion at the animal pole is a small trumpet- or funnel-like structure. A number of short villi (non-attaching filaments; average 200.3 ± 4.7/egg, $n = 38$) are distributed over the whole surface of the chorion. At spawning, eggs are held together in clumps by a tuft of long attaching filaments (average 29.6 ± 1.3/egg, $n = 38$) on the surface in the vegetal pole area of each egg (Iwamatsu, 1993).

A large, transparent yolk sphere is located in the center of each unfertilized egg. The cortical alveoli (vesicles, ca. 0.4–45 μm in diameter) and oil droplets are embedded at random in the cortical cytoplasm. The cortical alveoli contain a transparent colloidal material and usually one or sometimes a few spherical bodies (Iwamatsu and Ohta, 1976). The size of the oil droplets usually varies according to differences in the temperature during and after oocyte maturation, in the time after ovulation and among the individual females.

2.2. Stage 1 (3 min). Activated egg stage

When an egg is stimulated by s spermatozoon arriving at the vitelline surface through the micropyle, a transient wave of increase in cytoplasmic free calcium starts at the point of sperm attachment (Gilkey et al., 1978; Yoshimoto et al., 1986). The cortical alveoli in the vicinity of the micropyle also begin to break down (exocytosis of alveolar contents) about 9 s after sperm attachment (Iwamatsu et al., 1991). The wave of exocytosis begins to propagate over the whole egg surface and ends at the vegetal pole 154 s after its beginning. As a result of the exocytosis of cortical alveoli into the narrow space between the chorion and the vitellus, the chorion thins and hardens (Ohtsuka, 1960) as it separates from the vitellus to form a wide perivitelline space. Swollen spherical bodies secreted from the cortical alveoli are faintly visible in the perivitelline space. A transient 'contractile wave' of cortical cytoplasmic layer follows the wave of exocytosis (Iwamatsu, 1973; Iwamatsu and Hirata, 1984). Due to the oscillatory contractions following this distinct contractile wave, the cortical cytoplasm progressively accumulates toward the animal pole to form a thick cytoplasmic layer (Abraham et al., 1993; Sakai, 1964). At 7–8 min after sperm entry, the second polar body is extruded onto the surface of the cytoplasm at the center of the area where the germinal vesicle broke down during oocyte maturation.

2.3. Stage 2. Blastodisc stage

The male and the female pronuclei migrate toward and associate with each other at the center of the thick cytoplasmic disc at the animal pole. Both pronuclei fuse and form a zygote nucleus before breakdown of the nuclear envelope (Iwamatsu and Kobayashi, 2002). Chromosomes then appear and divide into two groups at the poles of the spindle marking the end of this stage.

a (30 min). Oscillatory contractions cause the peripheral cortical cytoplasm to migrate towards the animal pole where it forms a convex, lens-shaped blastodisc. Meanwhile, oil droplets are displaced toward the vegetal pole and begin coalescing.

b (60 min). The layer of cortical cytoplasm covering the yolk sphere is very thin except where it forms the cap-shaped blastodisc. By the end of this stage, most of the oil droplets from the animal hemisphere have already migrated to the vegetal hemisphere. Two dimple-like pits on the blastodisc serve as markers to locate the future blastomeres.

2.4. Stage 3 (1 h 5 min). 2 cell stage

The first cleavage plane is at a right angle to the axis between the second polar body (meiotic spindle) and the micropyle in 60–79% of eggs. The two blastomeres are highly rounded just after cleavage, but are comparatively flat just before the second cleavage.

2.5. Stage 4 (1 h 45 min). 4 cell stage

The second cleavage furrow develops on the two blastomeres at a right angle to the first cleavage plane. It deepens until each blastomere is divided into two of the same size. The oil droplets are larger but fewer and gather toward the vegetal pole.

2.6. Stage 5 (2 h 20 min). 8 cell stage

The third cleavage plane is parallel to the first and divides the four blastomeres into eight blastomeres. The blastoderm has bilaterally symmetrical rows of blastomeres and elongates along the axis of the second cleavage plane.

2.7. Stage 6 (2 h 55 min). 16 cell stage

The fourth cleavage plane, which is parallel to the second, divides the two rows of four blastomeres into four rows of four blastomeres.

2.8. Stage 7 (3 h 30 min). 32 cell stage

As a rule, the fifth cleavage plane divides the marginal 12 blastomeres meridionally into 24, and the central four blastomeres horizontally into eight thereby forming two layers, an outer and an inner layer, in the central region.

The number of marginal cells is 14 in most cases. These observations agree with those of Matui (1949), Gamo and Terajima (1963) and Iwamatsu (1976) but differ from the earlier reports of Kamito (1928) (cf. Yamamoto, 1975) in which cleavage was reported to continue to occur meridionally at least through the 32 cell stage.

2.9. Stage 8 (4 h 5 min). Early morula stage

The planes of the sixth and later cleavages are difficult to precisely trace. The blastomeres (64–128) have different cleavage planes depending on their positions within the dome-shaped blastoderm and are arranged in three layers. The peripheral blastomeres (21–24) are flattened in shape. The cells (30–35 μm in diameter) are arranged in 3–4 layers but are still easily dissociated from each other (Yokoya, 1966).

2.10. Stage 9 (5 h 15 min). Late morula stage

The blastodermal cells (256–512 blastomeres) are smaller than those of the previous stage and the number of marginal cells (30–40) has increased. The blastodermal cells (central regions, 25–35 μm in diameter) now form 4–5 layers.

2.11. Stage 10 (6 h 30 min). Early blastula stage

The blastoderm (about 1000 cells) is still high (thick) as in the late morula stage, although its inner cells (20–30 μm in diameter) are smaller. According to Kageyama (1987), the 11th cleavage still takes place synchronously. Nuclei from the marginal cells (40, cf. Kageyama, 1987) migrate out of the cells and are distributed in a few rows in the periblast (cortical syncytial layer).

2.12. Stage 11 (8 h 15 min). Late blastula stage

Projection of the underside of the blastoderm (central cells, about 20 μm in diameter) into the yolk sphere is observed. In this stage, some blastomeres begin to cleave asynchronously and to migrate (Kageyama, 1988). Several (5–6) rows of periblast nuclei are visible around the blastoderm.

2.13. Stage 12 (10 h 20 min). Pre-early gastrula stage

The blastoderm has flattened down onto the yolk sphere so that its outer surface follows the curvature of the yolk sphere. The cell layers are slightly thicker on one side. The diameter of the cells in the central region of the blastoderm remains about 20 μm.

2.14. Stage 13 (13 h). Early gastrula stage

The blastoderm begins to expand (epiboly, about 1/4 of the yolk sphere) over the surface of the yolk sphere, and the presumptive region of the embryonic shield arises as a thickened margin (dorsal lip) of the blastoderm. It is difficult to recognize the boundaries of the flattened marginal cells. The diameter of the cells in the central region of the blastoderm is 15–20 μm.

2.15. Stage 14 (15 h). Pre-mid-gastrula stage

Epiboly progressively advances and the blastoderm covers about 1/3 of the yolk sphere. The germ ring is well-defined, and the embryonic shield increases in size. Weak, rhythmically undulating movements (Fluck and Jaffe, 1988; Yamamoto, 1931) begin to occur on the blastoderm but not on the uncovered yolk sphere.

2.16. Stage 15 (17 h 30 min). Mid-gastrula stage

A streak is visible in the midline of the embryonic shield projecting into the germ ring area. The blastoderm covers about 1/2 of the yolk sphere. The nuclei of the marginal periblast are barely visible on the yolk sphere.

2.17. Stage 16 (21 h). Late gastrula stage

The blastoderm covers 3/4 of the yolk sphere, and the embryonic shield (body) becomes more clearly visible as a narrow streak. The enveloping layer expands uniformly over the yolk sphere until this stage (Kageyama, 1980).

2.18. Stage 17 (1 day 1 h). Early neurula stage (head formation)

The yolk sphere is nearly covered by the thin blastoderm leaving a small area around the vegetal pole (yolk plug) exposed. The head (rudimentary brain) is recognized anteriorly in the distinct embryonic body. A cell mass in front of the head rudiment exhibits a beak-like structure. A few small vacuoles (Kupffer's vesicles) appear at the underside of the caudal (posterior) end of the body, which is in contact with small blastopore.

2.19. Stage 18 (1 day 2 h). Late neurula stage (optic bud formation)

The brain and nerve cord in the arrow-shaped embryonic body develop as a solid rod of cells. A solid optic bud (rudimentary eye vesicle) appears on each side of the cephalic end. The beak-like cell mass is still visible. The Kupffer's vesicles enlarge somewhat. A small part of the yolk sphere still forms a blastopore at the vegetal pole.

2.20. Stage 19 (1 day 3 h 30 min). 2 somite stage

A groove appears in the dorsum of each optic lobe. At the end of this stage (three somites), two slight protuberant rudiments of otic (auditory) vesicles are recognized behind

the optic vesicles. The blastopore is completely closed. The expansion of the enveloping layer is accomplished without an accompanying increase in the number of constituent cells (Kageyama, 1980).

2.21. Stage 20 (1 day 7 h 30 min). 4 somite stage

A paired placode of otic (auditory) vesicles appears at the posterior region of the head. Depressions begin to form at the dorsal surface of the eye vesicles. Three parts of the brain (the fore-, the mid- and the hind-brain) are discernible.

2.22. Stage 21 (1 day 10 h). 6 somite stage (brain regionalization and otic vesicle formation)

The optic vesicles differentiate to form the optic cups and the lenses begin to form. The small otic vesicles appear, but they lack otolith. The three regions of the brain are well-defined, and the *neural fold* (*neurocoele*) is seen as a median line along the body. The flat body cavity is recognized on the surface of the yolk sphere bilateral to the mid-brain and hind-brain.

2.23. Stage 22 (1 day 14 h). 9 somite stage (appearance of heart anlage)

The tubular heart (heart anlage) appears underneath the head from the posterior end of the mid-brain to the anterior end of the hind-brain. The anlage of the hatching enzyme gland (cell mass) appears at the centroventral side of the hindbrain (Yamamoto, 1963), by migration of the cell mass, beak-like structure in front of the head rudiment (Inohaya et al., 1995). The body cavity extends further toward the posterior end of the eye vesicles. Melanophores appear on the yolk sphere in wild-type embryos. Incomplete lenses are present in the eyes, and the vesicular otocyst is defined.

2.24. Stage 23 (1 day 17 h). 12 somite stage (formation of tubular heart)

The anterior portion of the straight-tubed heart reaches beneath the posterior end of the eye vesicle. A pair of semicircular Cuvierian ducts (blood vessels) and the vitello-caudal vein begin to form on the yolk sphere. Kupffer's vesicles shrink. The neurocoele is formed in the fore-, mid- and hind-brains. The spherical optic lenses are completed. A blood island becomes pronounced in the ventral region between the 6th and 11th somites. The anterior (the 3rd to 5th) somites assume a slightly chevron-shape. The oil droplets have coalesced into a single large drop.

2.25. Stage 24 (1 day 20 h). 16 somite stage (start of heart beating)

The anterior portion of the heart, which exhibits a slow (about 33–64 min^{-1}) pulsation, extends up to the anterior end of the forebrain. Cuvierian ducts and the vitello-caudal vein are still incomplete. Kupffer's vesicles have almost disappeared. Otoliths are not yet present in the otic vesicles. The embryonic body encircles nearly 1/2 of the yolk sphere. The gut (digestive) tube is observed ventral to the somites.

2.26. Stage 25 (2 days 2 h). 18–19 somite stage (onset of blood circulation)

When blood circulation begins, the spherical blood cells are first pushed out of the blood island (7th to 15th somites) toward the vitello-caudal vein (Fig. 1). The blood is pumped (70–80 heartbeats/min) from the heart out into the anterior cardinal vein and the dorsal aorta roots. The dorsal aorta branching off the perceptible bulbus arteriosus is paired anteriorly with continuations extending to the head as the internal carotid arteries. The carotid artery splits to form the optic plexus, which connects with the left and right ducts of Cuvier. The left and right dorsal aorta roots run caudally until they join to form the dorsal aorta. The dorsal aorta is unpaired through the trunk region and continues into the tail as the vitello-caudal artery (Fig. 1). A countercurrent of the blood stream from the heart into the aorta is still observed.

Otoliths appear as two conglomerates of small granules lying against the inner surface of each well-expanded otocyst. The embryonic body encircles nearly 7/12 of the yolk sphere. The chevron-shaped somites are observed between the 3rd and 10th somites. Kupffer's vesicles have disappeared completely. The bulge of the liver anlage appears at the 1st–3rd somites just posterior to the future position of the left pectoral fin in the 19 somite stage.

2.27. Stage 26 (2 days 6 h). 22 somite stage (development of guanophores and vacuolization of the notochord)

Blood containing globular blood cells is pumped out beyond the anterior region of the hind-brain. The caudal vein is observed in the region from the 1st to the 14th somites. The tip of the tail is completely free of the yolk sphere. The anlage of the liver, which first appears at the 19 somite stage, is not yet well-developed. Red-brown colored guanophores, which first appeared at the ventral side of the mid-brain in the 20 somite embryo, are more clearly seen. Vacuolization of the notochord starts at its anterior region. Differentiating choriodea of the eyes begin to darken due to melanization.

2.28. Stage 27 (2 days 10 h). 24 somite stage (appearance of pectoral fin bud)

The tip of the tail where the notochord attaches is pointed. The embryonic body with the tail free from the yolk sphere encircles 5/8 of the yolk sphere. The rudiments of the pectoral fins protrude from the body trunk behind the base of the Cuvierian ducts. The eminences of liver rudiment are clearly seen on the left side beneath the 1st to 3rd somites, and the gut tube can be seen beneath

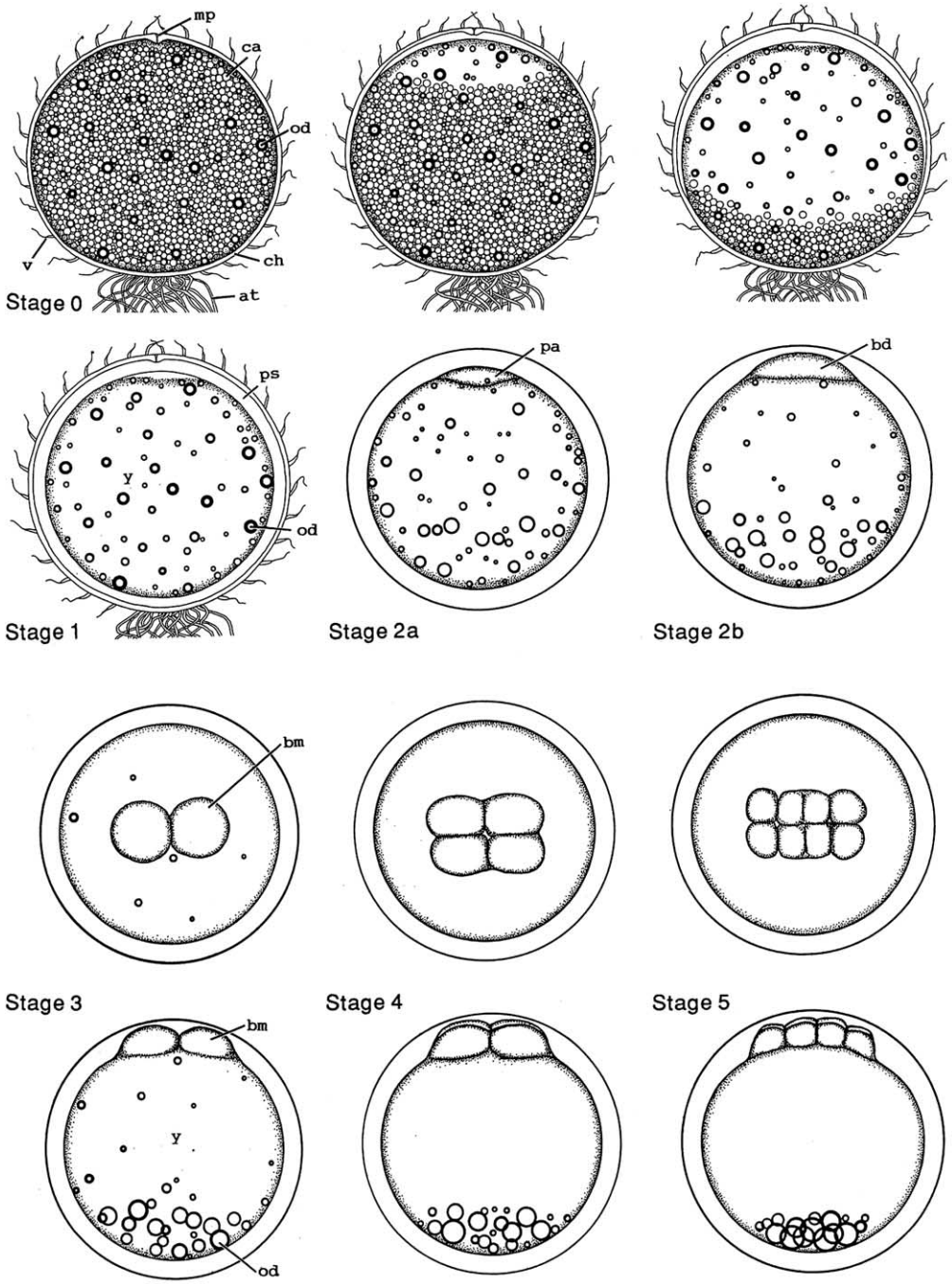

Fig. 1. ab, swim (air) bladder; af, anal fin; ag, artery globe; an, anus; at, attaching filament; bc, body cavity; bd, blastodisc; bi, blood island; bl, beak-like mass of cells; bm, blastomeres; br, branchiostegal ray; bv, blood vessel; ca, cortical alveolus; cd, Cuvierian duct; cf, caudal fin; ch, chorion; cn, cornea; cv, caudal vein; da, dorsal aorta; df, dorsal fin; dl, dorsal lip of blastopore; ea: otic (ear) vesicle; em, embryonic body; ev, otic (ear) vesicle rudiment; ey, optic (eye) vesicle; fb, fore-brain; fr, fin ray; g, gill; gb, gallbladder; gp, guanophores; gt, gut tube; h, heart rudiment; ha, atrium of heart; hb, hind-brain; hg, hatching gland cell; hv, ventricle of heart; kv, Kupffer's vesicle; l, lens; lj, lower jaw; lv, liver; mb, mid-brain; mc, marginal cell; mf, membranous fin (fin fold); ml, membrane labyrinth; mp, micropyle; mv, median yolk vein; n, naris; no, notochord; od, oil droplet; o, operculum; op, olfactory pit; ot, otolith; pa, protoplasmic accumulation; pb, protobrain; pf, pectoral fin; pi, pineal gland; pn, nucleus of periblast; pr, pronephros; ps, perivitelline space; s, scale; sc, spinal cord; sm, somite; sp, spleen; uj, upper jaw; uo, urinogenital orifice; v, non-attaching filament; vf, ventral fin; vl, vein of liver; y, yolk sphere.

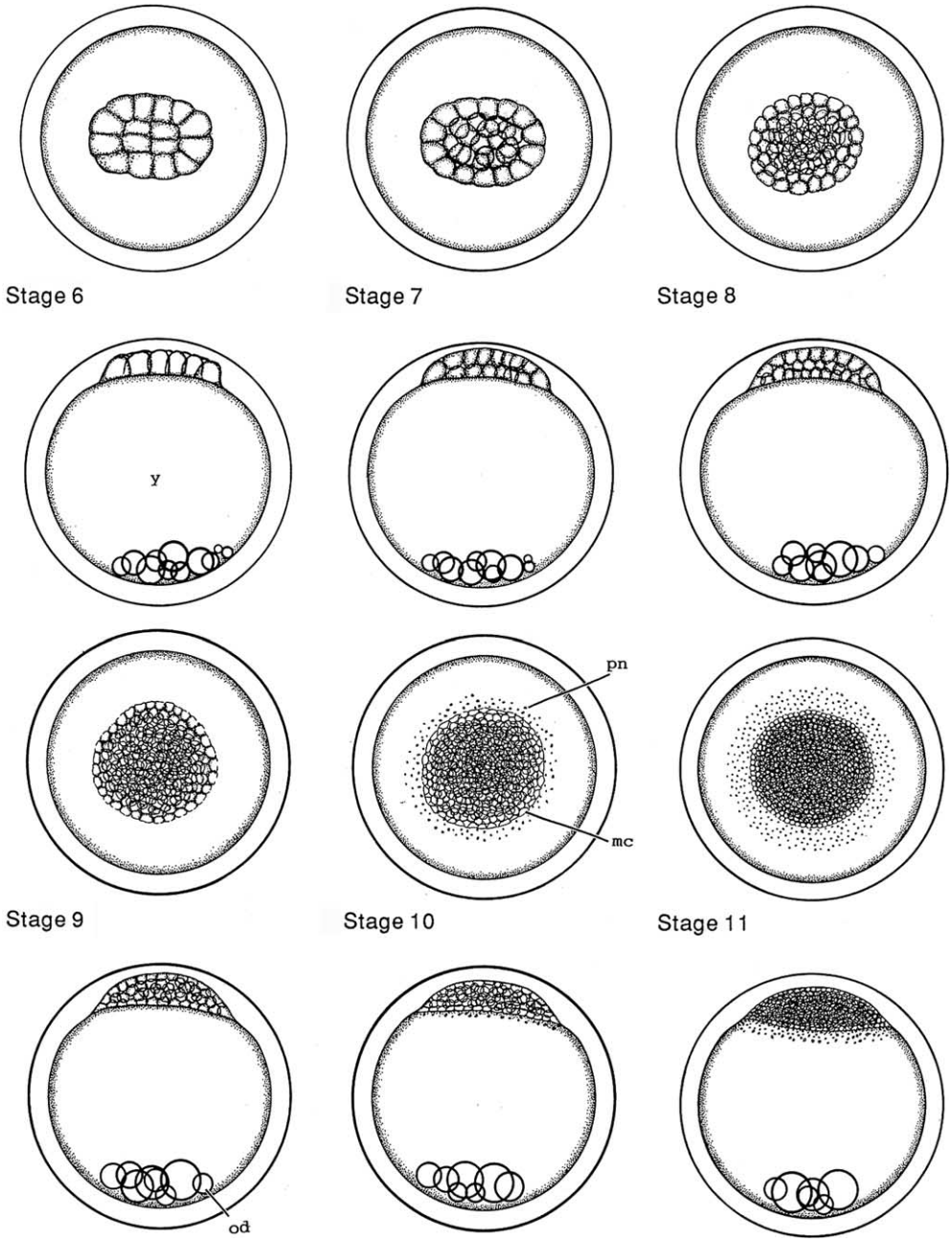

Fig. 1 (*continued*)

the 1st to 13th somites curving to the left-ventral in the region between the 1st and 3rd somites. The arterial end of the heart has shifted to the right. The tail is free of the yolk sphere, and its vein is observed from the 10th to the 16th somites.

2.29. Stage 28 (2 days 16 h). 30 somite stage (onset of retinal pigmentation)

The embryonic body with a caudal vein between the 10th and 22nd somites encircles about 2/3 of the yolk sphere. Pigmentation advances around the retina, and several melanophores occupy the dorsal wall of the viscera beneath the 1st to the 5th somites. The bulge of the liver becomes definitive in the left side of the 3rd to 4th somites. The anlage of the pancreas appears as a ventral eminence on the right side beneath and slightly anterior to the 3rd somite. Three sinuous portions of the vitelline veins consisting of the left and right ducts of Cuvier and four sinuous portions of the vitello-caudal vein meander on the yolk sphere. The blood cells (8.7 μm in diameter) flatten slightly. The posterior of the two otoliths in each otocyst is slightly larger than the anterior.

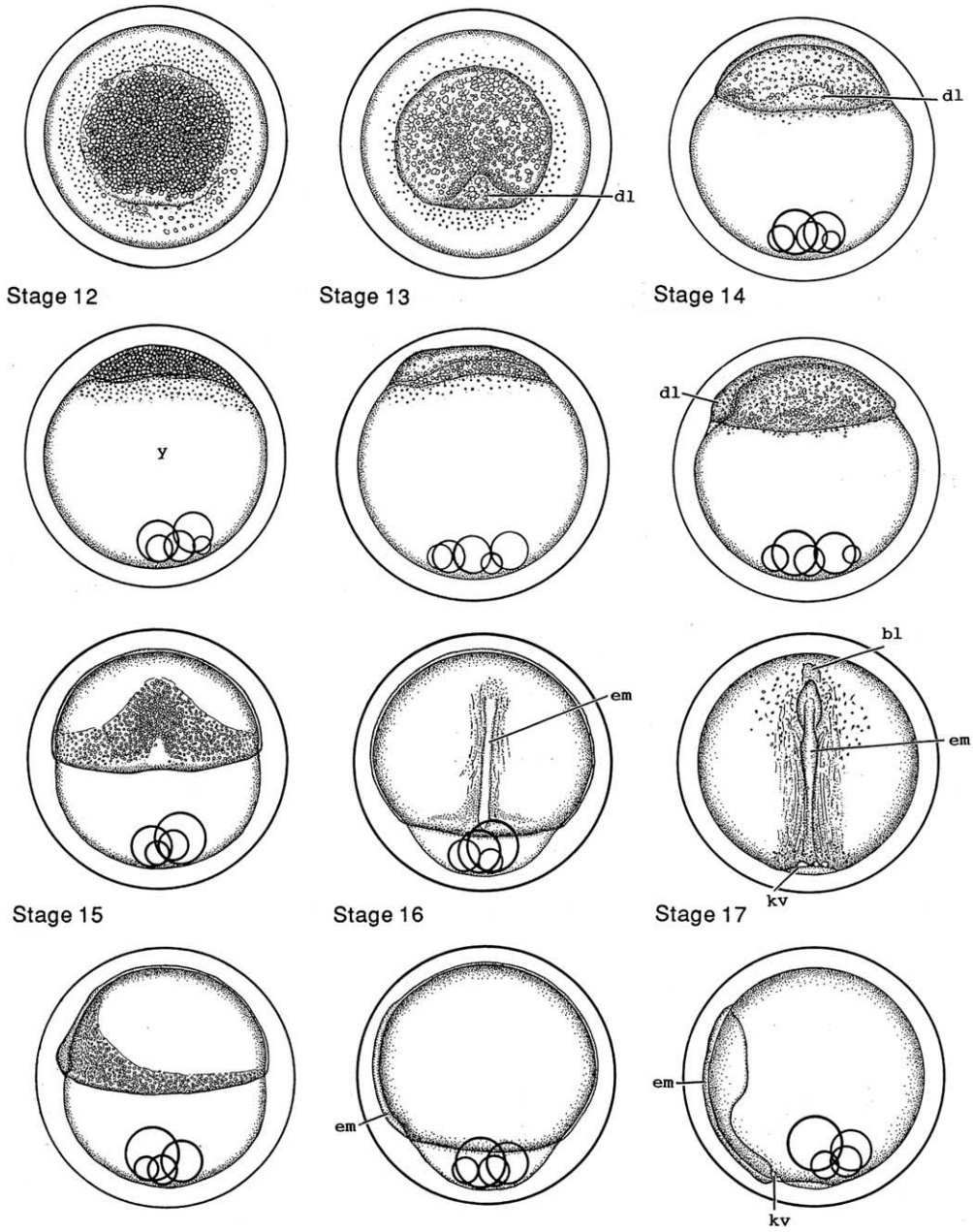

Fig. 1 (*continued*)

2.30. Stage 29 (3 days 2 h). 34 somite stage (internal ear formation)

The embryonic body encircles about 3/4 of the yolk sphere. The anlage of the pineal gland is recognized as a disc-shaped, round structure at the dorsal surface of the 3rd ventricle. In the heart, the sinus venosus, atrium, ventricle and bulbus arteriosus are differentiated. There is a large, transparent membranous protrusion inside the outer wall and another inside the inner wall of the otic vesicle (internal ear formation). In the region posterior to the eye (where gills will form), a group of large hatching enzyme cells has differentiated from endodermal cells (Ishida, 1944; Yamamoto, 1963). A ventral eminence is prominent behind the otic vesicles, and another eminence (the presumptive swim bladder) is discernible at the ventral side of the 3rd somite. The pectoral fin is apparent, and membranous fins (fin fold) are also seen in the tail, which has 19 somites beyond the gut tube. Guanophores begin to disperse on the dorsal surface of the body trunk. The anterior tip of the notochord is located where the branches of the dorsal aorta join.

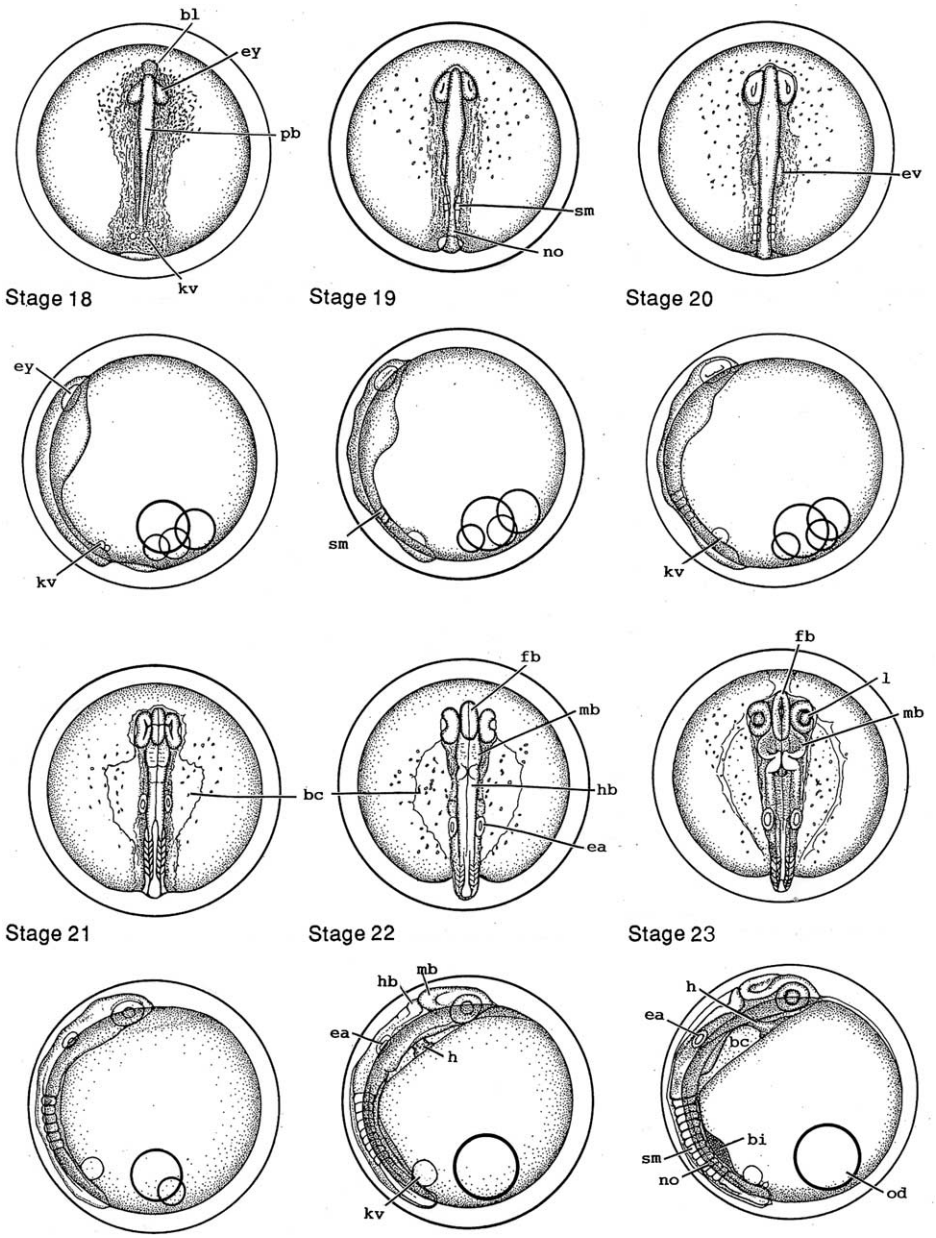

Fig. 1 (*continued*)

2.31. Stage 30 (3 days 10 h). 35 somite stage (blood vessel development)

The embryonic body covers nearly 5/6 of the yolk sphere. Branches of arteries supplying blood to the anterior musculature in the body trunk, the gills and the brain are observed. The hepatic vein of the liver drains into the left duct of Cuvier. Two transparent and membranous protrusions (will become semi-circular canals) are seen inside the outer wall of each otocyst. The swim bladder also becomes visible.

2.32. Stage 31 (3 days 23 h). Gill blood vessel formation stage

Large cells of the hatching enzyme gland migrate up to the region under the central part between the eyes, which now have a cornea. Blood circulation is seen in the gill arches. Pigmentation of the melanophores in the choriodea proceeds as a dark network in the eye. The pronephric kidney appears as a bright structure adjacent to the 1st somite. The transparent, colorless gallbladder first appears at the posterior region of the liver. Four transparent,

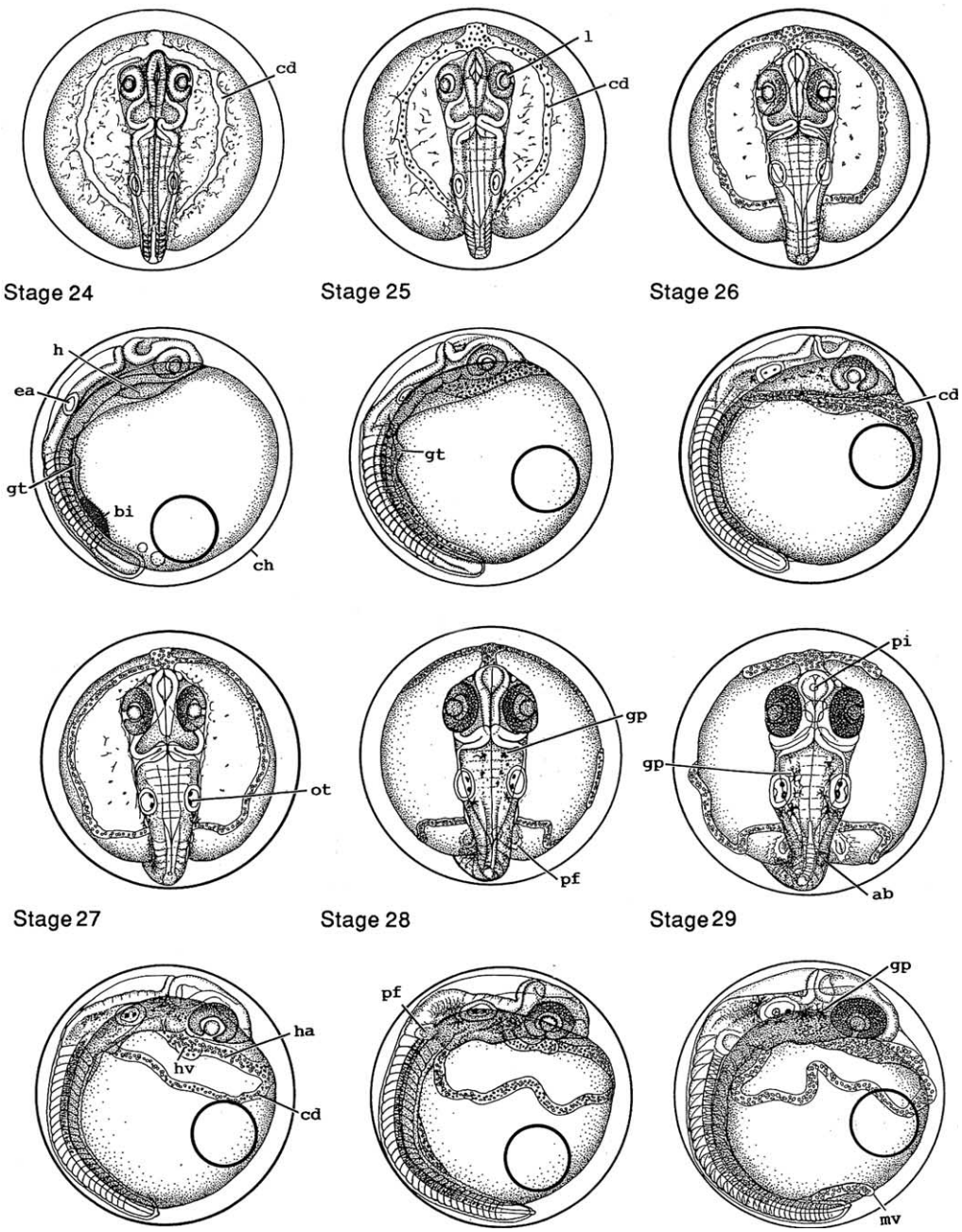

Fig. 1 (*continued*)

membranous protrusions (the structures of the internal ear) are recognized in the otic vesicles. The anterior region of the oral cavity is formed. The tail has 21 somites and a membranous fin which is wider on the ventral side.

2.33. Stage 32 (4 days 5 h). Somite completion stage (formation of pronephros and air bladder)

The swim (air) bladder is recognized as a transparent vacuolar body beneath the 3rd somite, and the distinct kidneys (pronephros) lie in contact with the bilateral sides of the notochord in the 1st somite. In the otic vesicles, a tubular (semi-circular canals) membranous labyrinth can be seen. In the posterior end of the tail, the somites are indistinct. The number of whole somites counted is 30. Two hours later, the blood stream is twisted in the posterior end of the tail.

2.34. Stage 33 (4 days 10 h). Stage at which notochord vacuolization is completed

The tail tip has not yet reached within interocular distance of the eye. Because the eyeball (choroidea) is

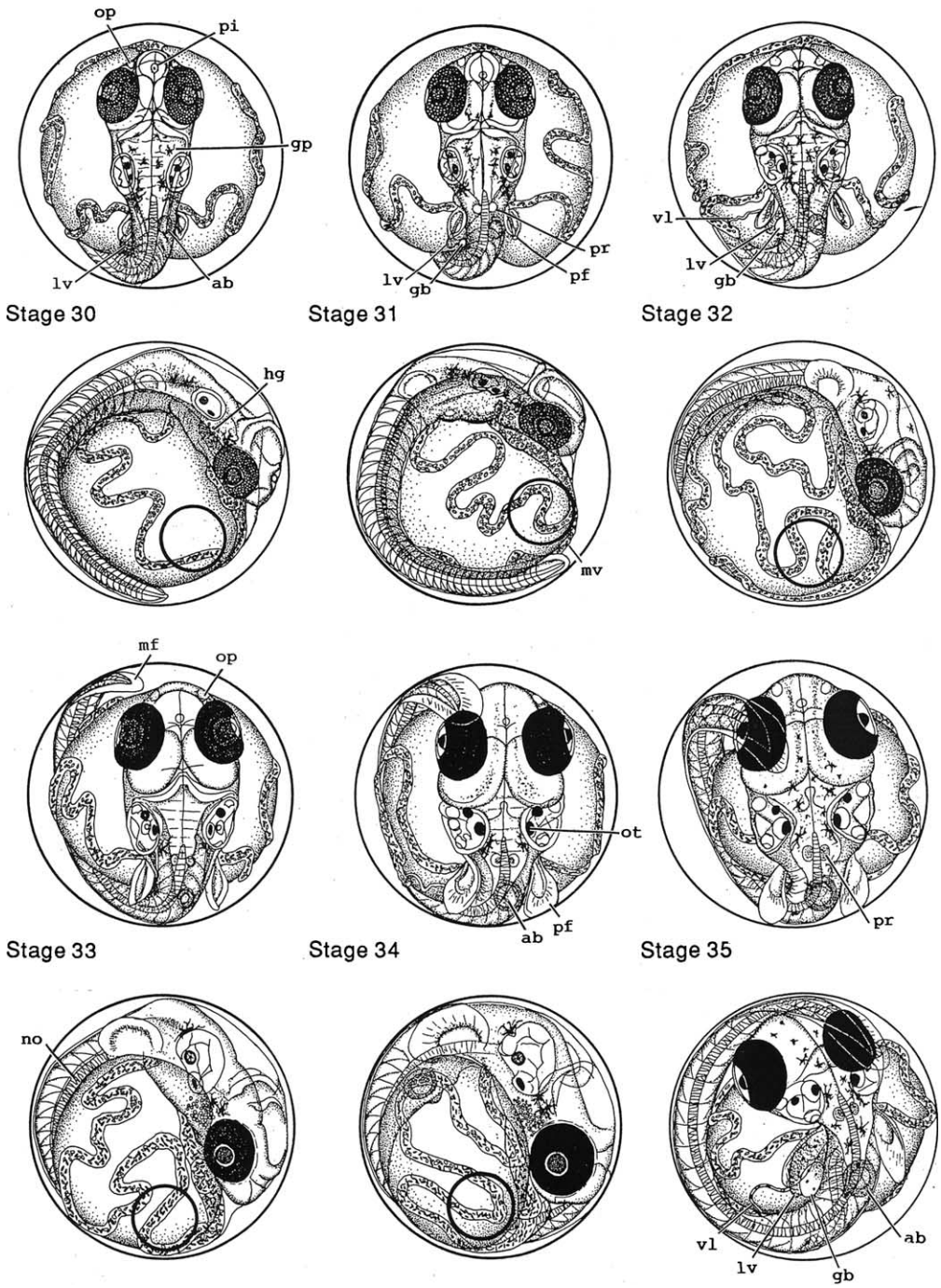

Fig. 1 (*continued*)

very dark, the lenses can be seen only with strong transillumination. The notochord is completely vacuolized to the end of the tail. The pineal gland is distinct at the dorsal surface of the vascularized forebrain. The tip of the membranous margins of the pectoral fins reaches the 4th somite.

2.35. Stage 34 (5 days 1 h). Pectoral fin blood circulation stage

The tip of the caudal fin has several melanophores and reaches the eye. Blood circulation is apparent in the pectoral fins, which frequently move (flutter).

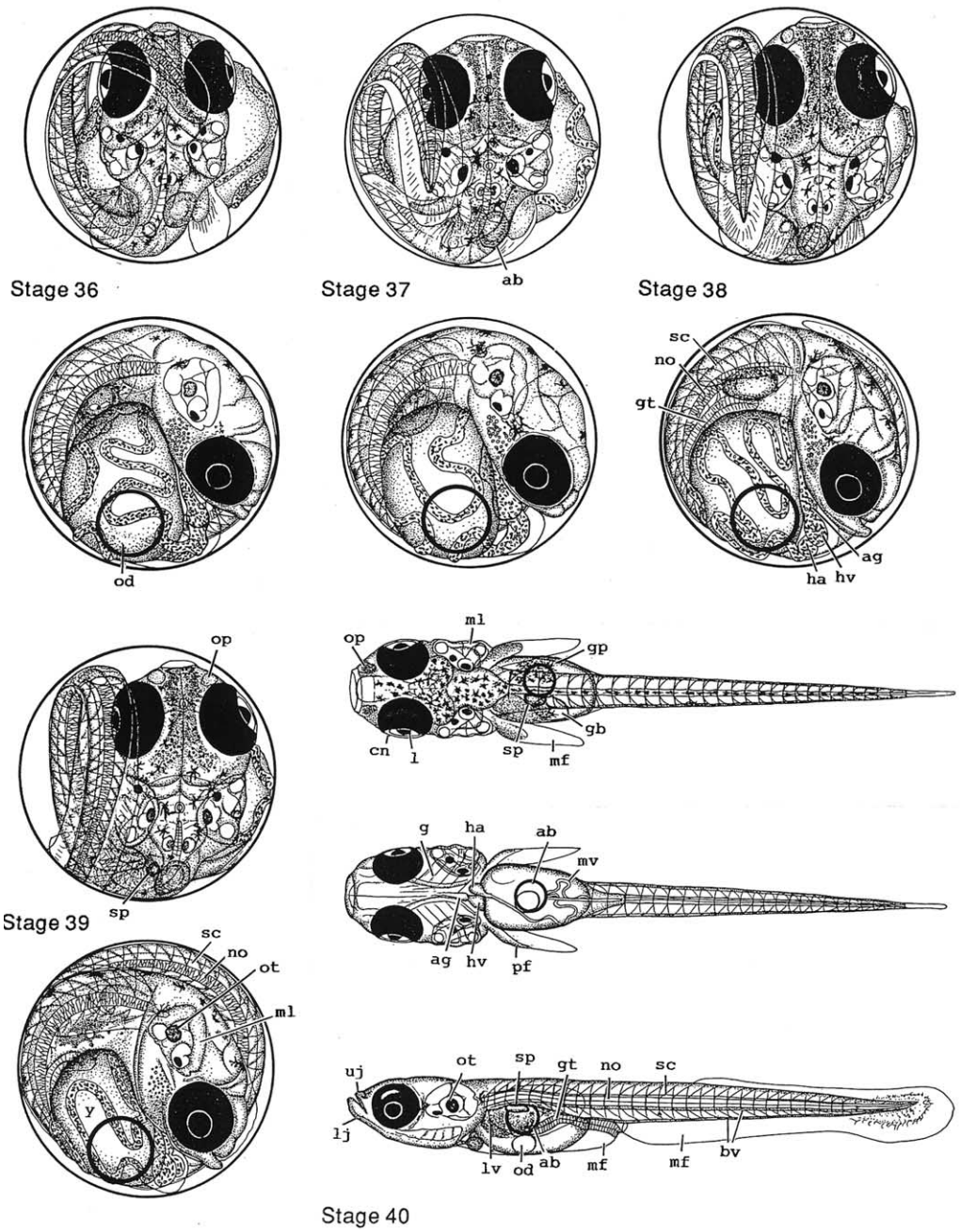

Fig. 1 (*continued*)

The choriodea of the eye becomes so black that it is almost impervious to light.

2.36. Stage 35 (5 days 12 h). Stage at which visceral blood vessels form

The tip of the caudal fin reaches beyond the posterior border of the eye. Guanophores are distributed from the head to the vicinity of the tail tip. Blood circulates through the internal tissues of the head and the viscera to Cuvier's ducts. The opening of the oral cavity to the mouth and the presence of several pit organs on the frontal bone can be recognized.

2.37. Stage 36 (6 days). Heart development stage

The tip of the tail reaches the otic vesicle. Guanophores and melanophores are distributed on the dorsal wall (peritoneum) of the peritoneal cavity beneath the 1st to the 4th somites. The extent of flexion of the atrio-ventricular region of the heart increases so that in a lateral view, the atrium and the ventricle lie adjacent to each other.

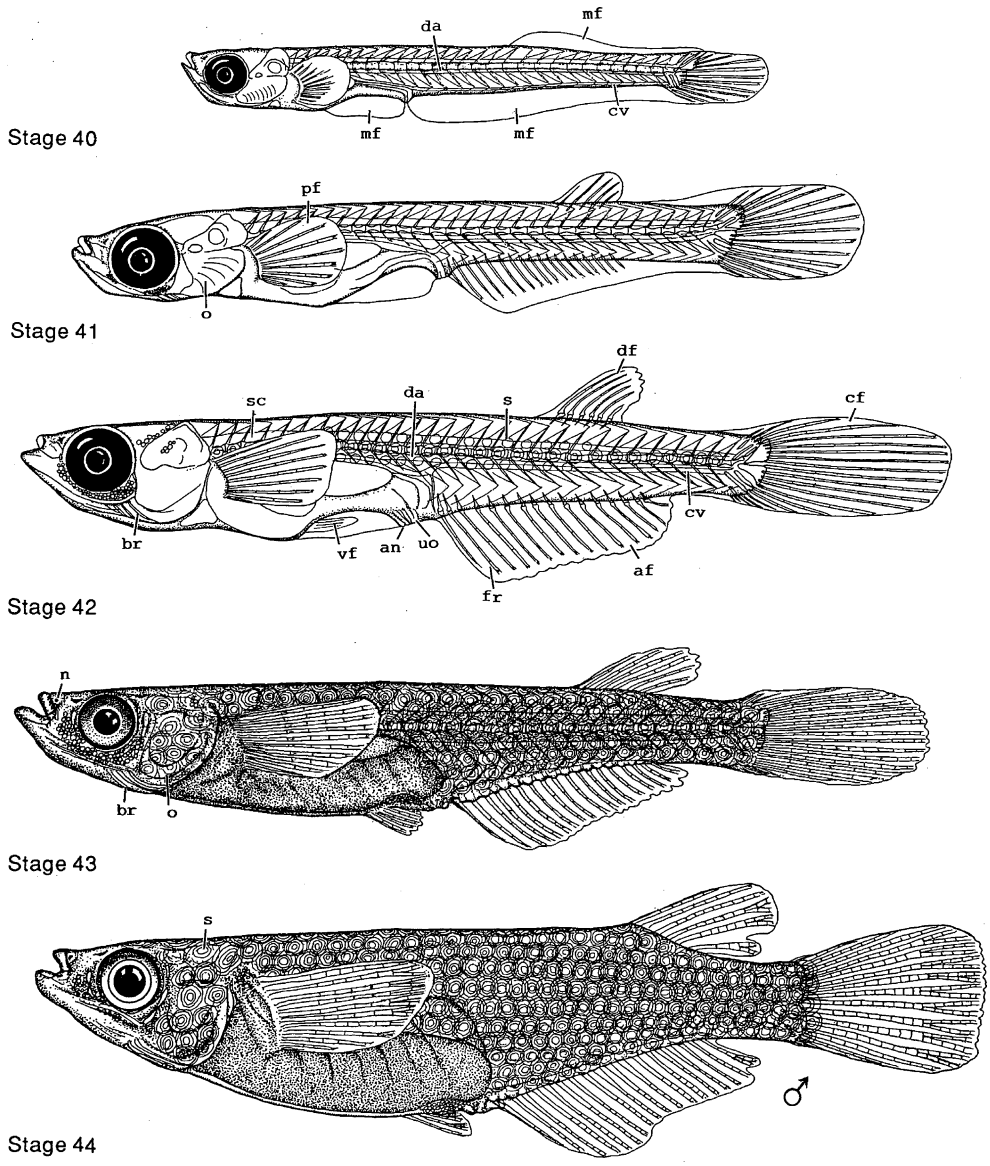

Fig. 1 (*continued*)

2.38. Stage 37 (7 days). Pericardial cavity formation stage

The tip of the tail lies just past the otic vesicle (ca. 3.1 mm total length; TL). The pharyngeal teeth are visible in the posterior region of the gills between the otic vesicles. The pericardial cavity (cardiac sac) surrounding the heart is easily observed. The slowly moving gut tube has a narrow lumen.

2.39. Stage 38 (8 days). Spleen development stage (differentiation of caudal fin begins)

The tip of the tail extends beyond the otic vesicle (ca. 3.6 mm TL), and the rudiments of the caudal fin rays can be seen within the round membranous fin. The spleen is recognized as a small reddish globule dorsal to the gut tube beneath the left region of the 3rd to 4th somites. The gut tube curves to the left between the 1st and the 4th somites, appearing to detour around the swim bladder (3rd to 4th somites). A large well-developed gallbladder can be identified by its yellow or yellowish green tint. Both eyes move actively at the same time accompanying movement of the mouth and the pectoral fins.

2.40. Stage 39 (9 days). Hatching stage

The tip of the tail extends to the base of the pectoral fin or to the posterior region of the swim bladder (3.8–4.2 mm TL). After hatching, the internal wall of the swim bladder expands remarkably.

Cells of the hatching gland have already disappeared. The embryos dissolve the inner layers of the chorion

(Yamagami, 1981), tear the single outer layer by moving the body and escape from the chorion tail-first.

2.41. Stage 40. 1st fry stage

This period extends from hatching until fin rays appear in the caudal and pectoral fins (about 4.5 mm TL).

2.42. Stage 41

This period begins after the first appearance of fin rays of dorsal and anal fins and continues until the appearance of ray nodes of caudal and pectoral fins, ribs in the trunk and neural spine on the vertebra (about 4.5–7.0 mm TL).

2.43. Stage 42

This stage follow parallel vascularization of the artery and the vein and extends to formation of the shape of all fins, complete vertebral column and the first appearance of the otolith (asteroscus) in the lagena, the scales and teeth on the upper jaw (about 7.5–10.0 mm TL).

2.44. Stage 43

This stage begins at the first appearance of ray nodes of dorsal and ventral fins and extends to the establishment of the number of fin rays of all fins and two rotations of the gut (about 10.5–15.5 mm TL).

2.45. Stage 44

This is the period from the formation of single dichotomous blanching at the distal end of fin rays of all fins to the appearance of the secondary sex characteristics such as urinogenital protuberance (♀) and papillar processes on fin rays (♂) (about 16.0–24.5 mm TL).

2.46. Stage 45

Three rotations of the gut and formation of double dichotomous blanching of the distal end of fin rays of all fins (more than 25 mm TL).

3. Experimental procedures

Mature *O. latipes* were purchased from a local fish farm (Yamato-koriyama, Nara Prefecture) and kept in freshwater in glass aquaria ($35 \times 30 \times 60$ cm^3) under artificial reproductive conditions (10 h dark, 14 h light; 26–28 °C). A measured amount of a powdered diet was supplied at least six times daily. The *Oryzias* spawn eggs every day about 9:00 (the onset of light). Ovaries with ovulated eggs in the ovarian lumen were routinely removed into saline (Iwamatsu, 1974) by laparotomizing females after pithing their brains. Unfertilized eggs were released from the ovarian lumen by tearing the ovarian sac. Long attaching filaments on each egg were carefully grasped with watchmaker forceps and cut off with the blunt tip of a glass bar while the egg was pulled away. Eggs were then transferred to a small petri dish containing saline and artificially inseminated by immersing them in a fresh sperm suspension in saline. The sperm suspension was prepared by squeezing sperm out of the testes of mature males.

Fertilized eggs were incubated in a stender dish (water depth 40 mm) first for 2 days in diluted (50%) sterile saline (26 ± 0.5 °C) containing methylene blue (2 ppm), and then in diluted saline without methylene blue. Eggs were observed using a special glass slide with a chamber (ca. 1 mm high) so constructed that the cover slip exerted a slight pressure on the chorion of the fertilized egg. By pushing the cover slip in various directions an egg could be rotated and the embryo could be held in any orientation. Each egg was used only once for observations. In some cases, it was necessary to dechorionate fertilized eggs by a convenient method (Iwamatsu et al., 1993) to allow observation of certain features.

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