

Stages of Embryonic Development of the Ice Goby (Shiro-uo), *Leucopsarion petersii*

Authors: Arakawa, Tomoko, Kanno, Yasuhiko, Akiyama, Nobuhiko, Kitano, Tadashi, Nakatsuji, Norio, et al.

Source: Zoological Science, 16(5) : 761-773

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zsj.16.761>

The BioOne Digital Library (<https://bioone.org/>) provides worldwide distribution for more than 580 journals and eBooks from BioOne's community of over 150 nonprofit societies, research institutions, and university presses in the biological, ecological, and environmental sciences. The BioOne Digital Library encompasses the flagship aggregation BioOne Complete (<https://bioone.org/subscribe>), the BioOne Complete Archive (<https://bioone.org/archive>), and the BioOne eBooks program offerings ESA eBook Collection (<https://bioone.org/esa-ebooks>) and CSIRO Publishing BioSelect Collection (<https://bioone.org/csiro-ebooks>).

Your use of this PDF, the BioOne Digital Library, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Digital Library content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne is an innovative nonprofit that sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

Stages of Embryonic Development of the Ice Goby (Shiro-uo), *Leucopsarion petersii*

Tomoko Arakawa¹, Yasuhiko Kanno^{2,3}, Nobuhiko Akiyama¹, Tadashi Kitano¹,
Norio Nakatsuji^{2†}, and Takako Nakatsuji^{1*}

¹*Tokai University School of Marine Science and Technology, Shimizu 424-8610, Japan,*

²*Mammalian Development Laboratory, National Institute of Genetics, Mishima 411-8540, Japan and*

³*Department of Regulation Biology, Faculty of Science, Saitama University,
Urawa 338-0825, Japan*

ABSTRACT—A series of normal stages for the embryonic development of the ice goby (shiro-uo), *Leucopsarion petersii*, which belongs to the Perciformes, is described. Stages are based on morphological features, by utilizing the optical transparency of live embryos from the first cleavage to the hatching stage. Fertilized eggs were obtained by artificial insemination and normal embryogenesis was accomplished in a defined medium in plastic petri dishes at 19°C. Shiro-uo eggs were surrounded by a very thin and clear chorion and could be dechorionated with forceps very easily. Developmental stages were mostly comparable to those of other fish embryos described so far, but several differences were indicated, such as the third cleavage plane being horizontal, and that the length of the cleavage cycle increased gradually from the very early stages. Also, there were differences in the relative rates of organogenesis of the brain, eyes, otic vesicles, and somites when compared to the zebrafish and medaka.

INTRODUCTION

The ice goby (shiro-uo in Japanese), *Leucopsarion petersii*, belongs to the family of gobies in the Perciformes group. The highly diverse Perciformes are the most numerous and advanced group of teleosts, but their embryogenesis has been examined rarely. Shiro-uo inhabits in coastal regions, and they enter rivers for spawning in coastal streams during January, along the Southern shore, to May, along the Northern shore of Japan (Matsui, 1986). This species is subject to fishing along the coast in many locations throughout Japan, and the caught fish are commercially dispatched and distributed as live animals to consumers. Mature adults of shiro-uo can be kept easily in fresh water and bred in the laboratory. The reproduction and ecology of shiro-uo have been reported in the field of fisheries science (Matsui, 1986; Akiyama and Ogasawara, 1994; Akiyama *et al.*, 1995). Our previous report of embryogenesis in this species was the first to describe the main features of embryonic development, including cleavage and epiboly (Nakatsuji *et al.*, 1997). The suitability of this fish for experimental studies, due to the total transparency of the whole embryo and ease of allowing normal development in a petri dish, was described. It also reported

that the first horizontal cleavage occurs between the 4-cell and 8-cell stages. Thus, the shiro-uo is a useful model for studying teleostean embryogenesis in the most advanced Perciformes group.

Definition of the normal stages is a fundamental step for studying embryonic development. The developmental stages of fish embryos have been studied by using various species such as *Fundulus heteroclitus* by Armstrong *et al.* (1965), *Gobius niger jazo* by Ballard (1969), *Austrofundulus myersi* Dahl by Wourms (1972), *Salmo gairdneri* and *Salvelinus fontinalis* (salmonid fishes) by Ballard (1973), *Amia calva* (holostean fish) by Ballard (1986), *Schliorhinus canicula* (lesser spotted dogfish) by Ballard *et al.* (1993), *Oryzias latipes* (medaka) by Iwamatsu (1994), *Danio rerio* (zebrafish) by Westerfield (1993), Kimmel *et al.* (1995) and Karlstrom *et al.* (1996). Among them, the descriptions of the zebrafish and medaka are the most detailed and suitable for use in developmental studies, and we used these studies as references for defining stages of the shiro-uo.

This report describes a series of stages for embryonic development of the shiro-uo, *Leucopsarion petersii*. Firstly, we defined the most suitable medium to allow normal development of chorionated or naked eggs in laboratory conditions. Then, developmental stages were defined, based on morphological features of the live embryos examined with ordinary transmission optics. Such stages were named according to the major developmental events rather than a number. Our description of the embryonic development from fertilization to

* Corresponding author: Tel. +81-543-34-0411;
FAX. +81-543-34-9837.

† Present address: Department of Development and Differentiation,
Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-
8506, Japan

hatching provides a basis for various experimental studies using the shiro-uo.

MATERIALS AND METHODS

Animals

Adult ice goby (shiro-uo), *Leucopsarion petersii*, which had been ascending a river for spawning when caught by fishermen at Hamashima in Mie Prefecture, Shimizu in Shizuoka Prefecture and Anamizu in Ishikawa Prefecture, were purchased in February, March and April respectively. They were transported in oxygenated packages with river water. The temperature was kept below 20°C during transportation. The body length of the mature shiro-uo was approximately 5 cm (Fig. 1). Adult females have clearly recognizable ovaries and their body size is larger than males. In addition, females have several dark spots aligned along the belly wall, while males do not have such spots.

Gonadal maturation and artificial insemination

Adult males were maintained in dechlorinated tap water at 18.0–19.0°C with air circulation and kept in the dark for 2 weeks to bring about gonadal maturation (Akiyama and Kitano, 1995). Matured testis was identified by examination through the transparent body wall (Fig. 1). Adult females were each injected with 25 units of gonadotropin (Teikokuzouki) and kept in dechlorinated tap water with air circulation at 18.0–19.0°C. Females that had ovulated were identified within 10 days, by the appearance of round eggs in the ovarian sac (Fig. 1). Such females were laid on ice for 1–2 min to give cold anesthesia. Ripe eggs were gently squeezed out from the belly of appropriate females into diluted (10%) sterile medium 199 (M199, Gibco) containing 25 mM Hepes buffer (pH 7.4), and mixed with sperm suspension obtained by mincing mature testes in the same diluted M199 solution. Such prepared unfertilized eggs could be used for fertilization at least for 5 hr, but the sperm suspension should be used within 10 min. Artificial insemination was completed within 30 min as indicated by the swelling and lifting of the chorion to an eggplant shape.

Incubation media for fertilized eggs and embryos

Fertilized eggs and embryos were incubated in sterilized plastic petri dishes at 19°C. The following different sterilized media were tested for supporting normal development; 10% M199 (a 9:1 mixture of distilled water and M199 containing 25mM Hepes buffer, supplemented with 100 units/ml penicillin and streptomycin), 50% M199, 100% M199, 50% M199 supplemented with 1, 2, 3, 5, 7, or 10 mM CaCl_2 , 10% artificial sea water (diluted Sealife, Marine Tech., whose ionic composition is almost the same as sea water), and tap water (dechlorinated with Tetra Contra Chlorine, Warner Lambert). The pH value of the 10% M199, 50% M199 and 100% M199 was 7.2–7.4. The pH of the 10% artificial seawater was around 7.0, and that of the dechlorinated tap water was 8.5–8.6. Fifty to 200 fertilized eggs were incubated in each medium and examined for normal development. In some experiments, the chorion was removed manually by fine forceps, and naked eggs were cultured in these media to find the best conditions to allow embryonic manipulation in future studies.

Standard developmental time

The normal development of shiro-uo was observed microscopically using approximately 500 fertilized eggs in total, after 4 separate artificial inseminations. The temperature was kept at 19°C throughout embryo development. The 0 hr was designated as the time when the chorion enlarged completely to give a long eggplant-shape and the blastodisc segregated from the yolk as a large and high mound-shaped cytoplasmic mass as described in the results section (Fig. 5a). We examined the time length between cleavage cycles. For this purpose, 8 embryos were evenly settled in a 60 mm plastic petri dish with 3 ml of the medium, so that each embryo could be identified by

marking on the dish bottom and examined continuously. Embryos showing any developmental abnormality were discarded. Time points were measured at the beginning of each cleavage from the 2-cell stage to the 64-cell stage. The time required to reach a particular stage was calculated as an average, using at least 20 embryos. Some of the data were statistically analyzed by the Student's *t*-test.

Microscopy

Photomicrographs of living embryos were taken by using an inverted microscope (Zeiss Axiovert 135) with transmission optics and an automatic camera. Diameter of blastomeres was measured by use of the photographic prints of embryos and a micrometer.

Microinjection of rhodamine into blastomeres

Microinjection was carried out by using a glass pipette (outer diameter of 0.5 μm , Femtotips, Eppendorf) connected to a microinjector (Narishige IM-5A and MN-151) under an inverted microscope (Nikon TMS-F). Injected solution was a dextran tetramethyl rhodamine (MW10,000) solution in PBS (12.5 mg/ml).

Histology

Embryos were fixed with 2.5% glutaraldehyde in 10% phosphate buffered saline (PBS) that was made by a 9:1 mixture of distilled water and PBS. 50% PBS was used for dechorionated embryos. Dehydration, paraffin embedding and sectioning was carried out using conventional methods. Sections were cut at 6 μm and were stained with Mayer's hematoxylin and eosin. Photomicrographs were taken using an Axioscop with an automatic camera (Zeiss).

Scanning electron microscopy

Embryos were fixed with 2.5% glutaraldehyde in 50% PBS, rinsed with PBS, and post-fixed with 4% osmic acid in 50% PBS. After dehydration through an ethanol series, embryos were transferred to *t*-butyl alcohol, and freeze-dried at -4 to -10°C . These samples were sputter-coated with gold and examined with a JEOL JSM5800/LV scanning microscope.

RESULTS

Selection of the incubation media.

Fig. 2a shows the rates of survival and normal development in various incubation media for 150 hr. Higher rates were obtained when fertilized eggs were incubated in 10% M199 or 10% artificial sea water (96.3% or 93.4%, respectively). Survival rates were lower in 50% M199 (48.4%) or 100% M199 (8.5%). Dechlorinated tap water was also not adequate as indicated by the survival rate of 34.3%.

For dechorionated embryos, 13 naked fertilized eggs were incubated in each of the various media. A 100% survival rate was observed in Hepes-buffered 50% M199 supplemented with 5 mM CaCl_2 and antibiotics (Fig. 2b). The same 50% M199 without CaCl_2 was less adequate with a survival rate of 53.8% if the chorion was removed at the 1-cell stage. Interestingly, the survival rate was 92.3% if the embryos were dechorionated at the 16- or 32- cell stage. Survival of dechorionated embryos decreased to 49.1% or 22.2% in 10% M199 or 10% artificial sea water, respectively, even when dechorionated at the 16- or 32-cell stage.

Accordingly, 10% M199 was used for incubation of shiro-uo embryos with an intact chorion, while 50% M199 supplemented with 5 mM CaCl_2 was used for dechorionated embryos in this study.

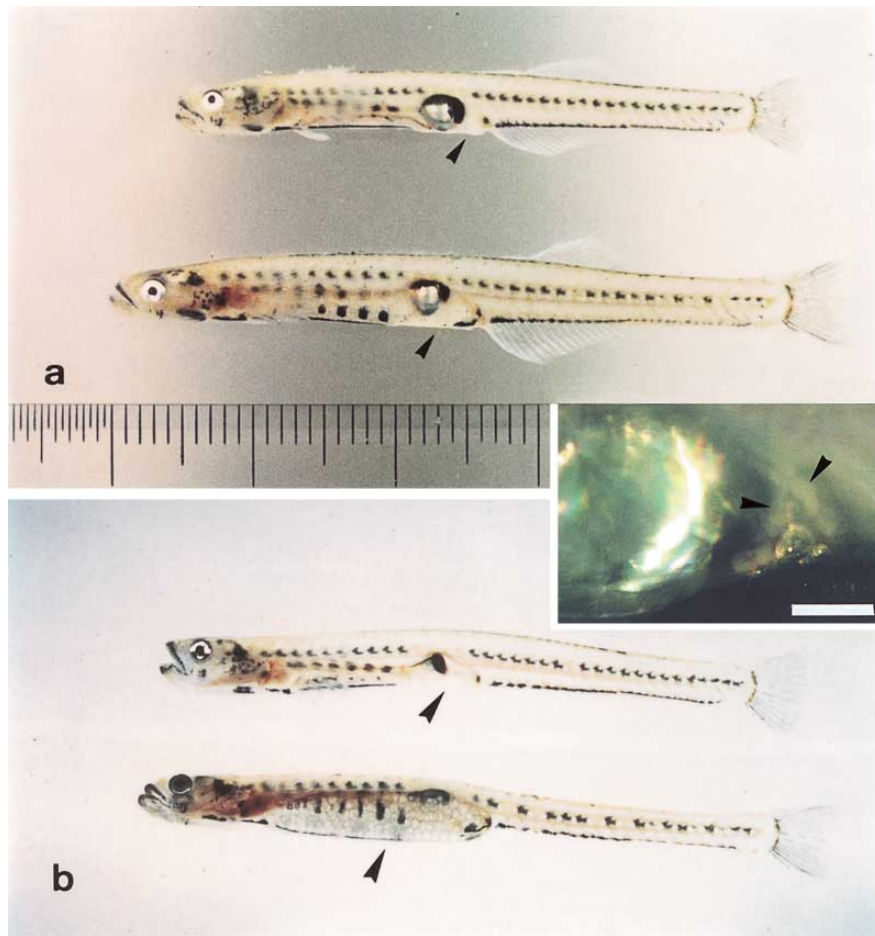


Fig. 1. Male (upper) and female (lower) shiro-uo before (a) and after (b) gonadal maturation. An inset shows an enlarged view of the male abdomen after gonadal maturation. Arrowheads indicate the testis or ovary. A scale bar in the inset indicates 100 μ m.

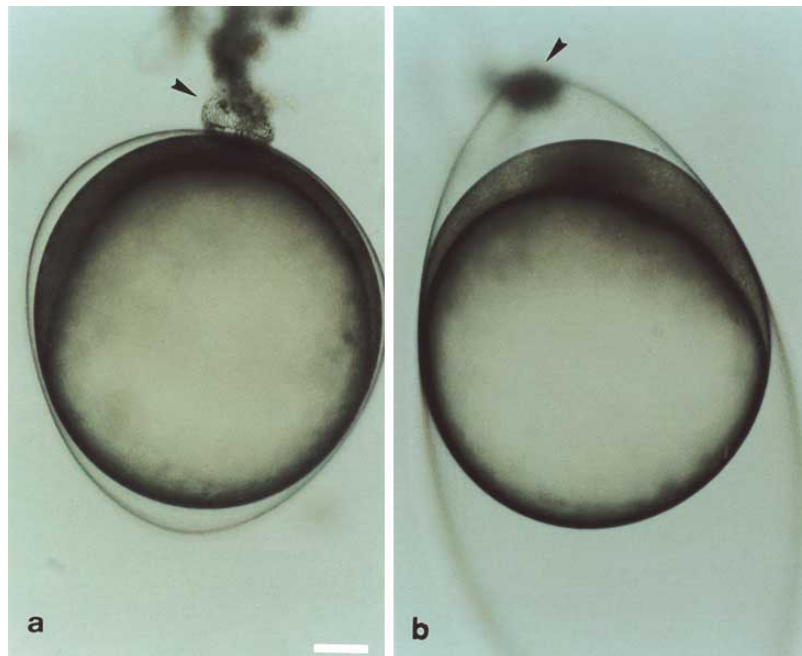


Fig. 3. Photomicrographs of an unfertilized (a) and a fertilized egg (b) at 20 min after insemination. A tuft of attachment filaments (arrowhead) was present on the chorion near the animal pole (upper side). Bar, 100 μ m.

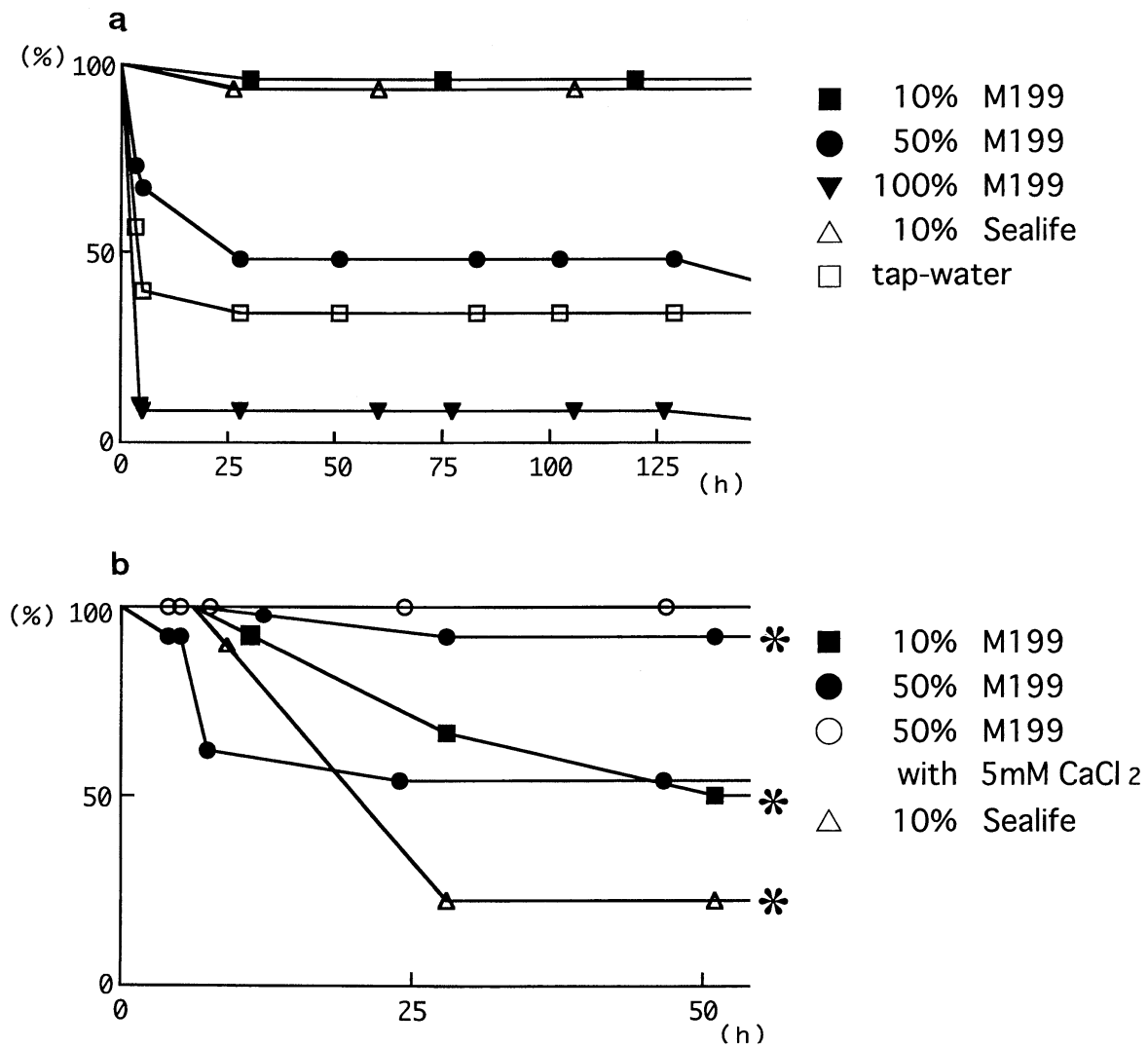


Fig. 2. Rates of survival and normal development of intact chorionated (a) or dechorionated (b) embryos in various incubation media. The chorion was removed at the 1-cell stage or at the 16- or 32-cell stage (lines with asterisk in b). Ordinate, % of normal embryos; abscissa, hr from insemination.

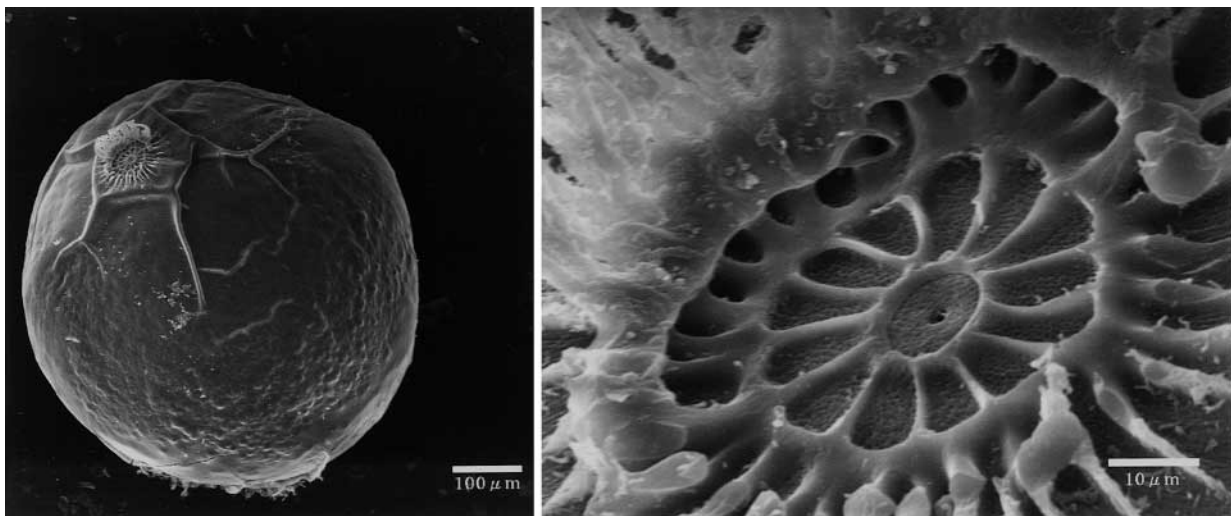


Fig. 4. Scanning electron micrographs of the micropyle on an unfertilized egg. Part of the attachment filaments was removed before fixation.

Fertilization

The chorion of unfertilized eggs was round and close to the egg surface but there was a recognizable perivitelline space (Fig. 3a). The time of fertilization was identified when the chorion began to swell to an eggplant-shape and the cytoplasm started to move toward the animal pole to form the blastodisc (Fig. 3b). As shown in Fig. 3a, a tuft of attachment filaments was present on the chorion near the animal pole. These filaments are used for attachment to the under side of rocks during spawning in rivers. The micropyle was located at the center of a tuft of attachment filaments, and was made of a distinctive radial structure with 13 petal-like repeats (Fig. 4).

Cleavage period

Two-cell stage (45 min)—The 1st cleavage furrow was oriented vertically. The furrow arose near the animal pole and progressed rapidly toward the vegetal pole to form 2 blastomeres of equal size. The cleavage furrow stopped at the yolk mass (Fig. 5b). This partial cleavage was also examined by making histological sections of the 2-cell stage embryo (data not shown). Also, an injection of rhodamin dye into a single blastomere indicated a free movement of the cytoplasmic fluid between the two blastomeres (data not shown). These blastomeres had a vertical diameter of approximately 240 μm and horizontal diameter of 320 μm .

Four-cell stage (1 hr 35 min)—The 2nd cleavage started near the animal pole at a right angle to the 1st cleavage furrow, forming 4 round blastomeres of equal size (Fig. 5c). The size of a blastomere at the 4-cell stage was approximately 250 μm in the vertical diameter and 220 μm in the horizontal diameter.

Eight-cell stage (2 hr 30 min)—The 3rd cleavage furrow appeared synchronously in the 4 blastomeres, in a horizontal orientation that divided them into approximately equal sizes (Fig. 5d). The resulting 8-cell stage blastodisc consisted of a

lower tier of 4 blastomeres attached to the yolk mass and an upper tier of 4 blastomeres which were separated from the yolk. However, there were a few occasions when the 3rd cleavage was not completely horizontal and the resulting 8 blastomeres were not arranged in two tiers but appeared as one tier.

16-cell stage (3 hr 30 min)—The 4th cleavage occurred vertically in the 8 blastomeres (Fig. 5e). However, in embryos where two regular tiers were not formed at the 8-cell stage, this cleavage occurred horizontally. Histological examination of 8- or 16-cell stage embryos showed the inner structure of the blastodisc (Fig. 6b). Blastomeres of the lower tier, which were attached to the yolk, extended their cytoplasm deeply into the yolk, while other blastomeres in the center of blastodisc were not attached closely to the yolk (Fig. 6b). Examination of similar embryos by scanning electron microscopy showed a 3-dimensional view of the blastodisc (Fig. 6a).

32-cell stage (4 hr 40 min)—The 5th cleavage occurred in the 16 blastomeres and formed 32 blastomeres arranged in approximately 3 tiers (Fig. 5f).

64-cell stage (5 hr 55 min)—The 6th cleavage occurred in the 32 blastomeres. During the following cleavages, the plane of cell division was randomized and synchrony was gradually lost.

Cleavage cycles

We examined the time length between cleavage cycles (Fig. 7). The average length and standard deviation of the 2nd cycle (2-cell to 4-cell stage) was 51 ± 7 min. The 3rd, 4th, 5th and 6th cycles were 53 ± 11 , 60 ± 8 , 67 ± 11 and 72 ± 7 min, respectively, as shown in Fig. 7. Although there was a large variation in the cycle length at each stage, this analysis indicated a gradual but statistically significant increase of the cycle length from the earliest cleavage stage to the 6th cycle. For example, the 2nd cycle was significantly shorter than the 4th

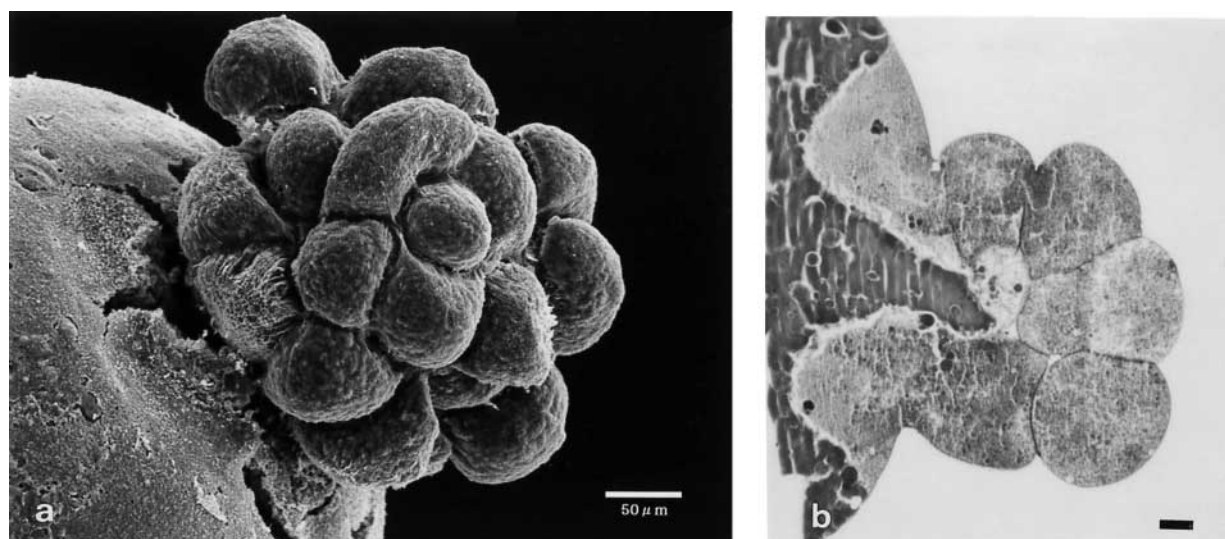


Fig. 6. A scanning electron micrograph (a) and a histological section (b) of the 16-cell stage embryo. Bar, 50 μm .

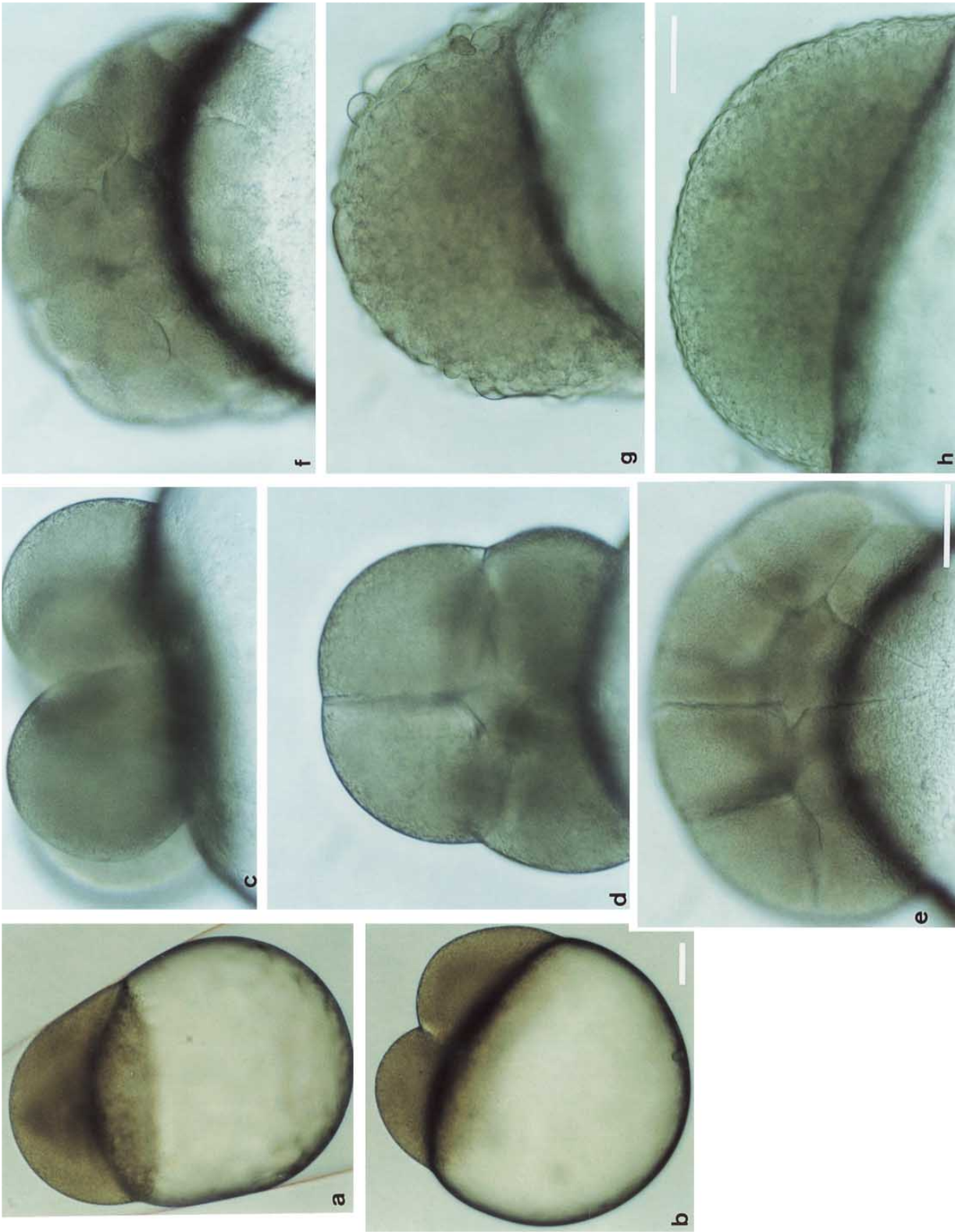


Fig. 5. Photomicrographs of embryos during the cleavage period and morula and blastula period at the 1-cell stage (a), 2-cell stage (b), 4-cell stage (c), 8-cell stage (d), 16-cell stage (e), 32-cell stage (f), morula stage (g), and blastula stage (h). Bars, 100 μ m; a–b, c–e, and f–h are at the same magnification respectively.

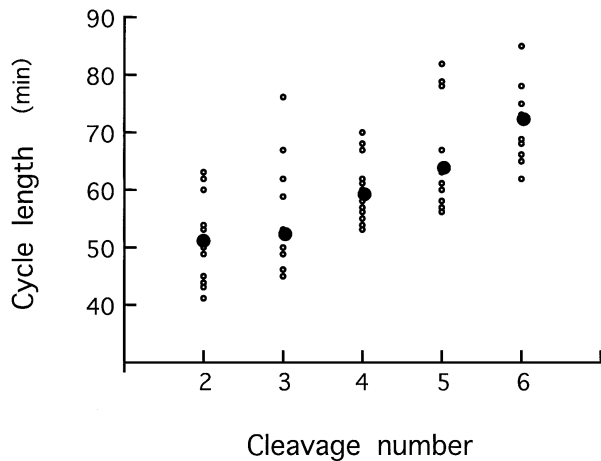


Fig. 7. A histogram showing the time length between cleavage cycles during the early cleavage period. The average value is shown by the larger dot for each cycle.

cycle ($p < 0.05$) or the 5th and 6th cycles ($p < 0.01$) by Student's *t*-test.

Morula and blastula period

Morula stage (7 hr)—Further cleavages occurred several times in randomized planes, at approximate intervals of 60–90 min during the morula period. The whole blastoderm maintained a high mound-shape until 12 hr after fertilization (Fig. 5g).

Blastula stage (15 hr)—Around 15 hr after fertilization, the blastoderm started to flatten down to the yolk mass (Fig. 5h). The peripheral cells along the blastoderm surface transformed from a round to a flattened epithelial shape. An embryo shown in Fig. 8a and b represents the stage shortly before the initiation of epiboly (17 hr).

Epiboly period

30%-epiboly stage (20 hr)—The base of the blastoderm transformed from a flat plane into a concave shape, and the circumference of the blastoderm started an epibolic movement along the yolk surface. If the focus of the microscope was adjusted to the margin of the blastoderm on the yolk surface, the advancing front line of the superficial cell layer was observed clearly. We followed the previous report by Kimmel *et al.* (1995) where the early gastrula stage was defined as 30%-epiboly, when the blastoderm margin was located 30% of the distance between the animal and vegetal poles (Fig. 8c, d).

50%-epiboly stage (24 hr)—The blastoderm had covered half of the yolk sphere. This stage is equivalent to the mid-gastrula stage. The embryonic shield was defined clearly (Fig. 8e, f) and the germ ring at the marginal region had thickened. The yolk mass was deformed by the compressing force created along the front line of the advancing superficial cell layer. The germ ring on the dorsal side, corresponding to the posterior end of the embryo, had advanced further toward the vegetal pole than the ventral side.

90%-epiboly stage (26 hr)—The engulfing movement of the blastoderm and embryonic shield over the yolk mass had progressed further (Fig. 8g, h).

100%-epiboly stage (27 hr)—Epiboly came to an end as the blastoderm had completely covered the yolk mass, indicating 100%-epiboly (Fig. 8i).

Bud stage (30 hr)—The embryonic shield had enlarged, and the neural plate had thickened along the embryonic axis. Prospective head and tail regions were recognizable along the anterior-posterior axis (Fig. 8j). The whole embryo had turned in the chorion so that the head was now directed downward away from the attachment filaments of the chorion. The body length was approximately 0.8 mm. The yolk had transformed from a sphere to an oval-shaped mass (Fig. 8j).

Segmentation period

Three-somite stage (39 hr)—The 1st somite had formed and was followed by the 2nd somite, posterior to the first, at around 37hr after fertilization. At 39 hr, the 3rd somite was added to the posterior side (Fig. 9a). A pair of rudimentary eye vesicles appeared in the cephalic region. The brain and spinal cord were enlarged and the Kupffer's vesicles became visible. The body length was 1.0–1.1 mm.

Nine-somite stage (48 hr)—The forebrain, midbrain and hindbrain became discernible from the 6-somite stage. A small groove appeared in each of the optic lobes (Fig. 9b). The Kupffer's vesicles were enlarged, and the yolk mass had become elliptical. The body length was 1.2 mm.

11-somite stage (53 hr)—A pair of rudimentary otic vesicles was discernible. The tail tip had extended and detached from the yolk mass. The body length was approximately 1.3 mm.

14-somite stage (59 hr)—The shape of the somites had changed to become a V-shaped structure aligned along the trunk (Fig. 9c) and the otic vesicles had formed clearly in the hindbrain region. The three brain regions were well defined. The tail had extended prominently and blood islands had become visible. The Kupffer's vesicles had disappeared and the vitellocaudal vein had begun to form on the yolk mass. The body length was 1.5 mm.

20-somite stage (70 hr)—The optic vesicles had developed to form the optic cups, and the lenses had begun to form. The primordial heart was visible as a tubular structure underneath the head region, between the mid-brain and hind-brain.

24-somite stage (80 hr). The lenses had become prominent. The tail was well extended and slightly curved (Fig. 10a). The anus and gut tube were discernible and a regular heart-beat started at the 25-somite stage. The body length was 1.8–2.0 mm.

30-somite stage (4.5 days)—The retina began to be pigmented and blood circulation in the aortic arch was observed. The ventricle and atrium had developed within the heart. The tail had extended further and straightened.

32-somite stage (5 days)—Strong blood circulation and tail motility were observed. The retina showed network-shaped

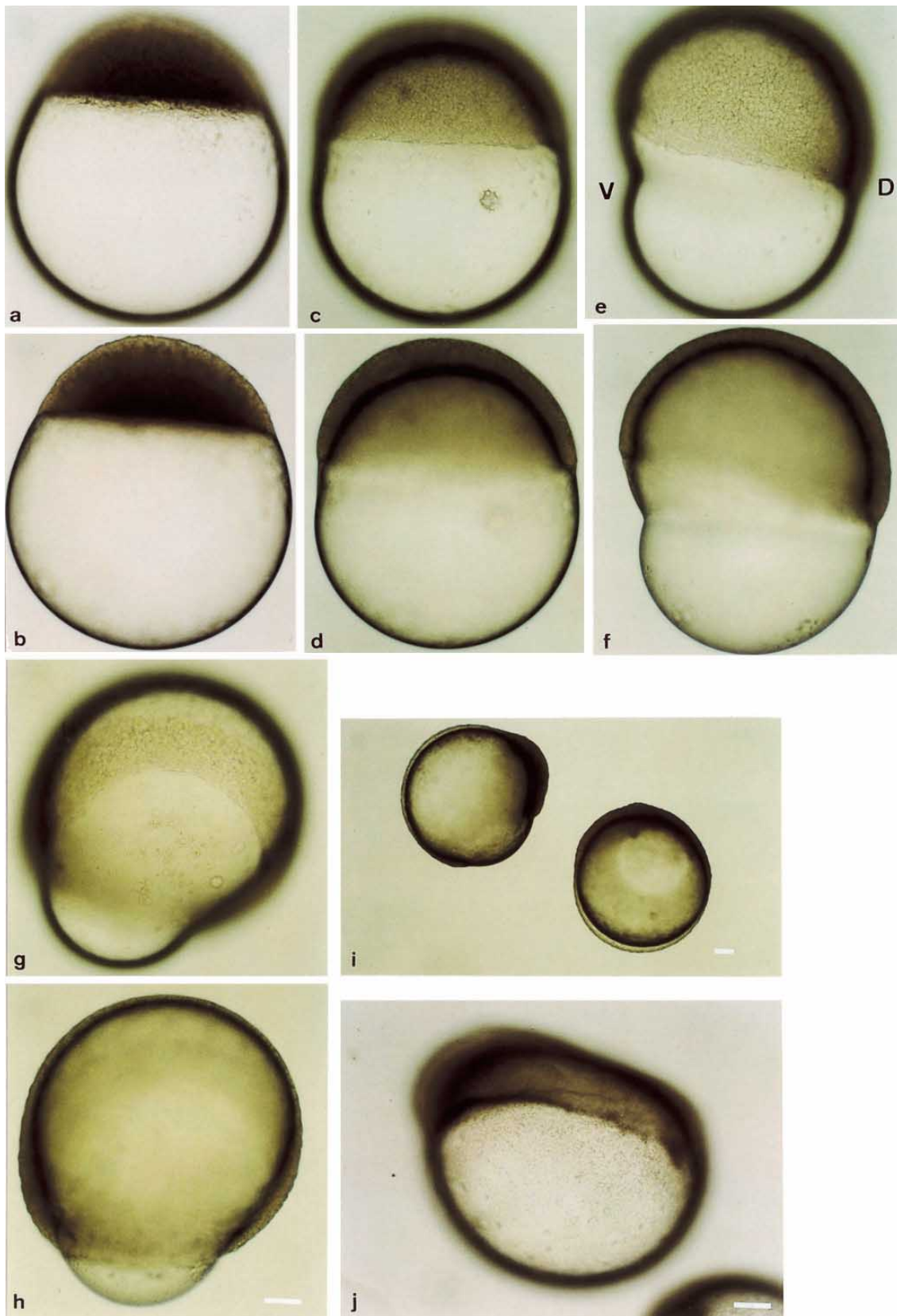


Fig. 8. Photomicrographs of embryos during the epiboly period. A representative embryo at late blastula stage (**a**, **b**), 30%-epiboly stage (**c**, **d**), 50%-epiboly stage (**e**, **f**), 90%-epiboly stage (**g**, **h**). For each pair of photographs, the upper photograph was focused on the blastoderm surface and the lower photograph was focused on the center. Embryos at 100%-epiboly stage (**i**) and bud stage (**j**) are also shown. Bars, 100 μ m; **a–h** are at the same magnification.

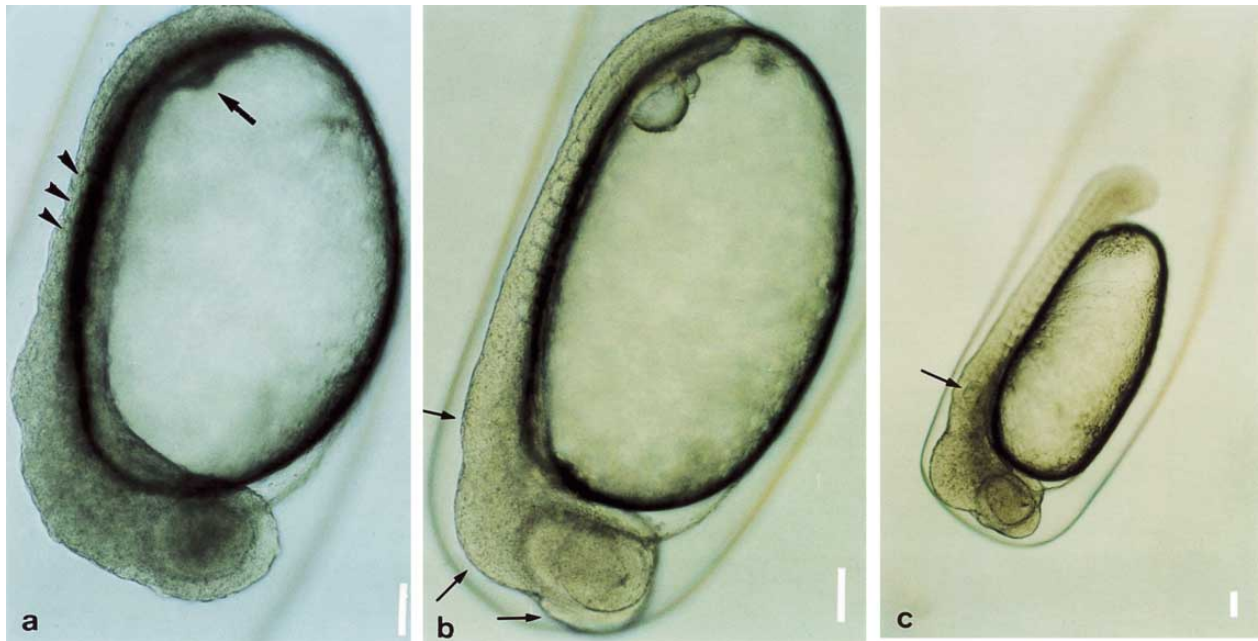


Fig. 9. Photomicrographs of embryos during the segmentation period. (a) A 3-somite stage embryo with somites (arrowheads), eye vesicles, and Kupffer's vesicles (arrow). (b) A 9-somite stage embryo with discernible forebrain, midbrain and hindbrain (arrows). (c) A 14-somite stage embryo with V-shaped somites and otic vesicles (arrow). Bars, 100 μ m.

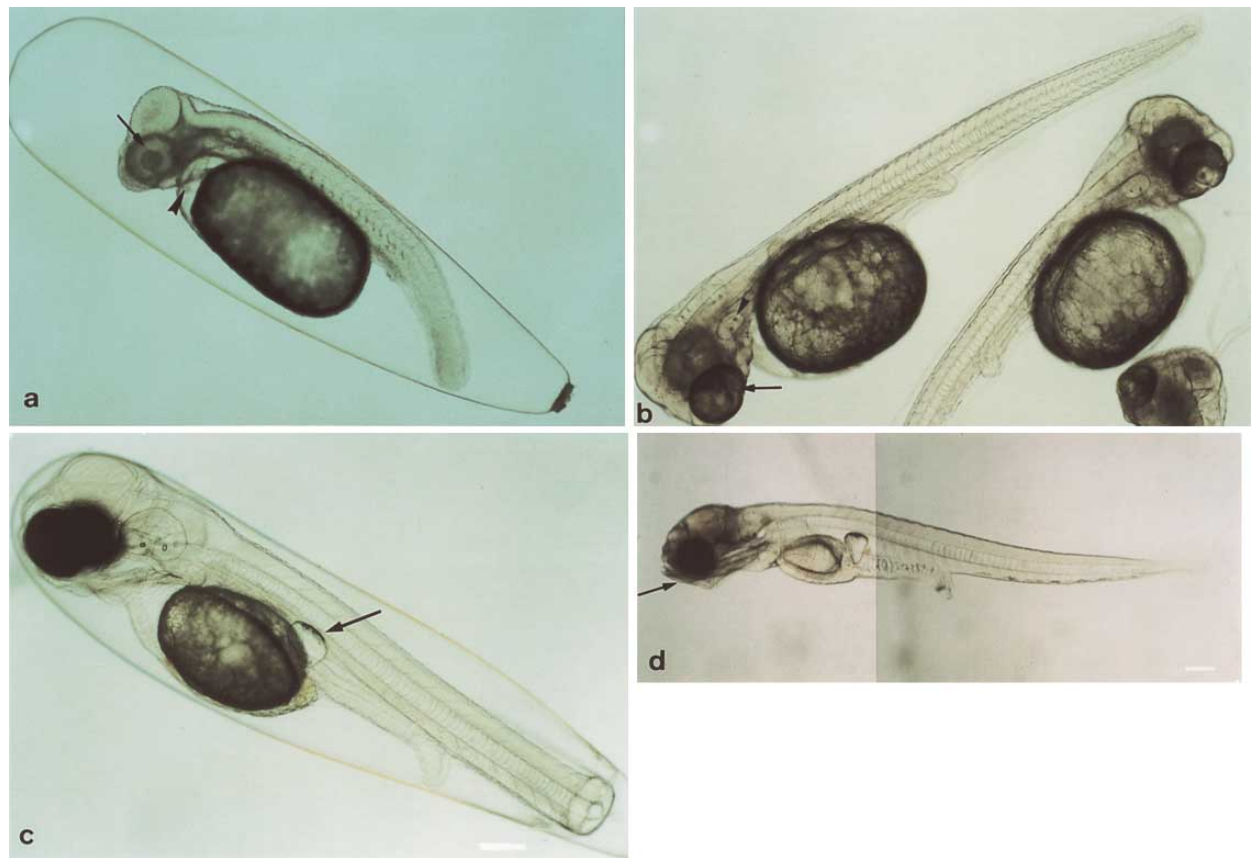
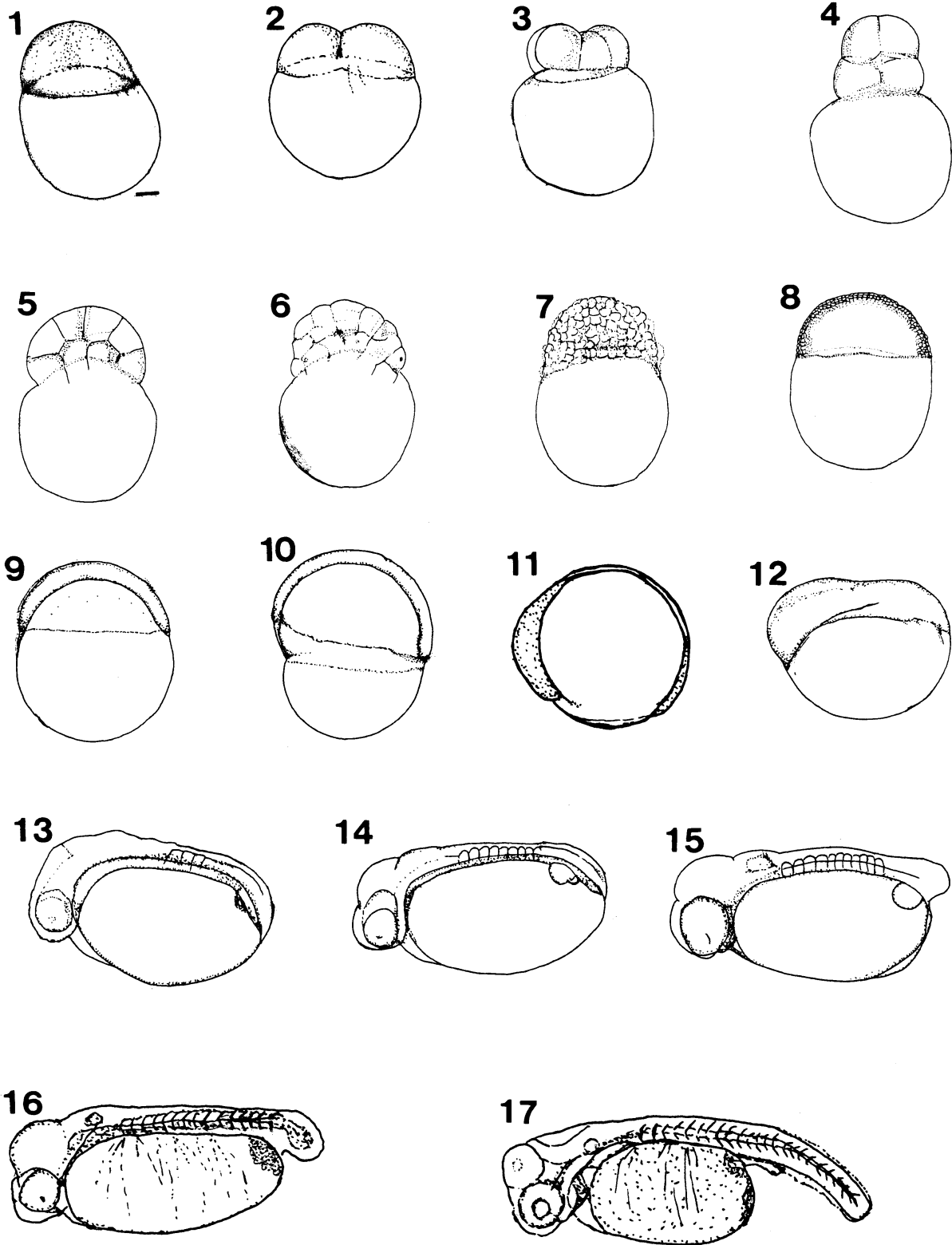


Fig. 10. Photomicrographs of later stage embryos. (a) A 24-somite stage embryo with lenses (arrow) and heart (arrowhead). (b) A 32-somite stage embryo with pigmented retina (arrow) and otoliths inside the otic vesicles (arrowhead). (c) A 10 days old embryo with very dark eyes and swimming bladder (arrow). (d) A 12 days old embryo with a distinct lower jaw (arrow). Bars, 200 μ m; a–c are at the same magnification.



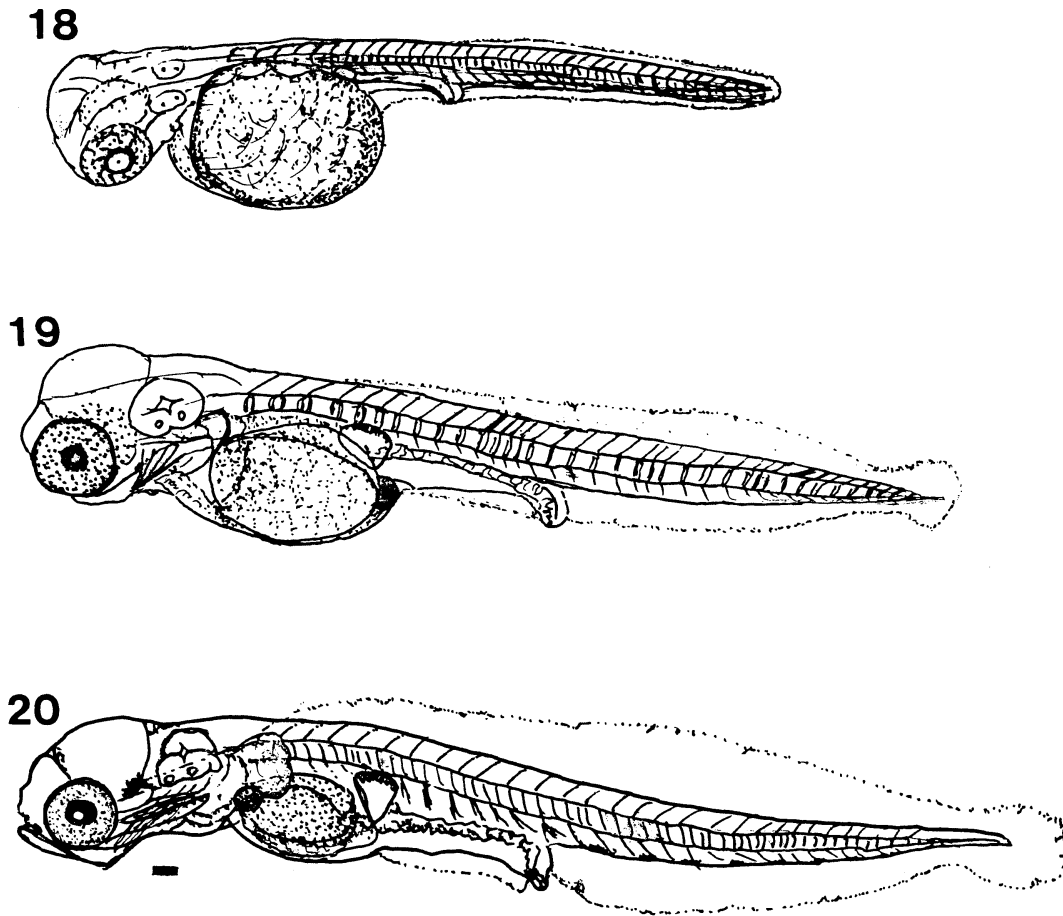


Fig. 11. Sketches of the shiro-uo embryo at selected stages, which are indicated by the sketch number in Table 1. Scale bars in sketches at the first and last stage indicates 100 μ m.

pigmentation and the otoliths were clearly visible as two conglomerates of granules in the well-developed otic vesicles (Fig. 10b). The body length was 2.8 mm.

36-somite stage (6 days)—Formation of the somites was completed. The pectoral fins had begun to form.

Later development—At 7 days, the gallbladder had become a greenish-yellow due to the bile. At 8 days, the mouth had begun to open occasionally. At 9 days, the spleen and liver had become visible. At 10 days, the eyeballs had become very dark, and the blood circulation had become red due to the presence of hemoglobin. The otic vesicles had become larger. The swimming bladder was recognizable as a transparent vacuolar organ. The liver, pancreas, gallbladder and kidneys were well developed (Fig. 10c). The distinctive kidneys and pronephroi were located in contact with the bilateral sides of the notochord. The pectoral fin and tail had become increasingly motile and the tail was either well extended or folded (Fig. 10c). The body length was 3.4 mm.

At 12 days, the eyes, mouth, pectoral fins and tail moved actively. The lower jaw was well differentiated (Fig. 10d). The body length was 3.9 mm. Hatching occurred 13 days after fertilization. Hatched larvae retained their transparency. The body length was 4.3 mm, and the yolk had almost disappeared

by this stage. Progress of the developmental stages of shiro-uo is summarized in Table 1.

DISCUSSION

It is necessary to find adequate conditions to ensure normal embryogenesis of fertilized eggs of shiro-uo, with or without an intact chorion, to allow experimental manipulation in future studies. Our present results indicated that 10% M199 or 10% artificial seawater was the best medium for incubation of the intact embryos. For dechorionated embryos, the best medium was Hepes-buffered 50% M199 supplemented with 5 mM CaCl_2 , which enabled a survival rate of 100%. The same 50% M199 without CaCl_2 was less adequate, with a survival rate of 53.8% if the chorion was removed at the 1-cell stage. Most of the embryonic abnormality and death was caused by detachment of the blastomeres from the blastoderm or yolk mass. The addition of 5 mM CaCl_2 apparently increased cell adhesiveness and repressed cell detachment. We tested several concentrations of CaCl_2 , and found that 5 mM was the most suitable. If dechoriation was carried out at the 16 or 32-cell stage, the survival rate was improved to 92.3%, even when such embryos were cultured in 50% M199 without CaCl_2 .

Table 1. Progress of the developmental stages of shiro-uo embryos at 19°C

| Period, stage (body length) | Time | Notes | Sketch No. |
|-----------------------------------|-------------|----------------------------------|------------|
| Zygote 1-cell | 0 hr 0 min | | 1 |
| <i>Cleavage period</i> | | | |
| 2-cell | 0 hr 45 min | | 2 |
| 4-cell | 1 hr 35 min | | 3 |
| 8-cell | 2 hr 30 min | | 4 |
| 16-cell | 3 hr 30 min | | 5 |
| 32-cell | 4 hr 40 min | | 6 |
| 64-cell | 5 hr 55 min | | |
| <i>Morula and blastula period</i> | | | |
| morula | 7 hr | | 7 |
| blastula | 15 hr | | 8 |
| <i>Epiboly period</i> | | | |
| 30%-epiboly | 20 hr | | 9 |
| 50%-epiboly | 24 hr | | 10 |
| 90%-epiboly | 26 hr | | |
| 100%-epiboly | 27 hr | | 11 |
| bud stage (0.8 mm) | 30 hr | turning inside chorion | 12 |
| <i>Segmentation period</i> | | | |
| 3-somite (1.0–1.1 mm) | 39 hr | eye vesicles, Kupffer's vesicles | 13 |
| 9-somite (1.2 mm) | 48 hr | forebrain, midbrain, hindbrain | 14 |
| 11-somite (1.3 mm) | 53 hr | otic vesicles, tail bud detached | 15 |
| 14-somite (1.5 mm) | 59 hr | blood islands | 16 |
| 20-somite | 70 hr | optic cups, lenses, heart | |
| 24-somite (1.8–2.0 mm) | 80 hr | heartbeat | 17 |
| 30-somite | 4.5 d | retina pigment, circulation | |
| 32-somite (2.8 mm) | 5 d | tail motility, otoliths | 18 |
| 36-somite | 6 d | pectoral fins | |
| <i>later development</i> | | | |
| | 7 d | gallbladder bile | |
| | 8 d | mouth opening | |
| | 9 d | spleen, liver | |
| (3.4 mm) | 10 d | hemoglobin, swim bladder | 19 |
| (3.9 mm) | 12 d | active motility, lower jaw | 20 |
| hatching (4.3 mm) | 13 d | | |

This may suggest that the adhesiveness between blastomeres increases from the 16- or 32-cell stage.

The micropyle of shiro-uo eggs was surprisingly located at center of a tuft of attachment filaments on the chorion at the animal pole, and it consisted of complicated radial structures. In many teleost species, the micropyle is located on the opposite side from the attachment filaments. This unusual feature may be related to the fact that shiro-uo eggs are attached to the under side of rocks during spawning in rivers.

The developmental stages of the shiro-uo were comparable to most other fish embryos described so far, but the present study revealed a few differences when compared to the medaka and zebrafish (Iwamatsu, 1994; Kimmel *et al.*, 1995). Firstly, the 3rd cleavage furrow appeared horizontally in the 4 blastomeres in the shiro-uo embryo. Therefore, the resulting 8-cell stage embryo consisted of a lower tier of 4 blastomeres attached to the yolk mass and an upper tier of 4 blastomeres which were separated from the yolk. In contrast, two regular tiers appear much later in the zebrafish and medaka, at the 32-cell stage. Embryogenesis of another *Gobius* species reported so far (Ballard, 1969) also has one-layered blastomeres until 16-cell stage. Secondly, the time length between cleavages showed a significant increase dur-

ing the early cleavage period. In most species however, the early cleavage cycles have a constant length until the mid-blastula transition. In fact, cleavage cycles in medaka and zebrafish have a fixed time length of 35 min and 15 min respectively, during the early cleavage period (Iwamatsu, 1994; Kimmel *et al.*, 1995).

During early epiboly, the marginal zone advanced toward the vegetal pole at the same pace to keep radial symmetry of the blastoderm, but later, the germ ring on the dorsal side was more advanced toward the vegetal pole than on the ventral side, which created an asymmetrical deformation of the yolk mass. Such a difference in the epiboly rate on the dorsal and ventral sides has not been reported in the medaka (Kageyama, 1980) and zebrafish (Kimmel *et al.*, 1995). There is no such fact described either in case of *Gobius niger jazo* (Ballard, 1969). Also, the yolk mass was transformed from a sphere to an oval-shaped mass at the bud stage, and later to an elliptical mass. In the case of medaka and zebra fish embryos, the yolk mass remains spherical throughout embryogenesis.

During the segmentation period, various organs of shiro-uo embryos showed different rates of development when compared to the medaka and zebrafish (Iwamatsu, 1994; Kimmel

et al., 1995). The optic buds and Kupffer's vesicles appeared at the 3-somite stage in shiro-uo embryos, while they appear at the 5-somite stage in the zebrafish or at the bud stage in medaka. The forebrain, midbrain and hindbrain became discernible at the 6- to 9-somite stage in shiro-uo and medaka embryos, while they are formed at around the 13-somite stage in the zebrafish. In shiro-uo embryos, the otic vesicles appeared at the 11-somite stage and the lenses began to form at around the 20-somite stage. However, the otic vesicles and lenses appear at the 6-somite stage in medaka embryos. Zebrafish embryos have otic vesicles at the 10-somite stage and lenses at around the 21-somite stage. The otolith appeared at the 30-somite stage in the shiro-uo, at the 26-somite stage in the zebrafish and at the 18-somite stage in the medaka. A regular heartbeat was observed at the 25-somite stage in the shiro-uo, while it is seen at the 16-somite stage in the medaka, and at the 27-somite stage in the zebrafish. In the present study, we observed a total somite number of 36 in shiro-uo embryos. Akiyama and Kitano (1995) however, have reported 36-38 somites in the shiro-uo. Zebrafish embryos have a total somite number of 30-34, and medaka embryos have 30-35 somites.

Recently, vigorous efforts have been concentrated to investigate the developmental processes of fish embryos using the zebrafish and medaka. However, it is also significant to study embryogenesis in other teleost species, especially those belonging to evolutionary distant groups. The zebrafish, *Danio rerio*, belongs to the Cypriniformes group, while the medaka, *Orizias latipes*, is a member of the Atheriniformes. In contrast, the shiro-uo, *Leucopsarion petersii*, belongs to the Perciformes group, which is the most numerous and advanced group of teleost. In this report, we have described several advantages of the shiro-uo for experimental studies, such as the ease of artificial insemination, normal development of intact and dechorionated embryos in simple laboratory conditions, and total transparency of embryos. These features make the shiro-uo an appropriate model for the study of teleostean embryogenesis.

REFERENCES

- Akiyama N, Ogasawara Y (1994) Reproductive behavior of the ice goby *Leucopsarion petersii* in captivity. *Suisanzoshoku* 42: 577-584 (in Japanese)
- Akiyama N, Kitano T (1995) Egg development of Ice-goby, *Leucopsarion petersii*, by artificial fertilization. *Rep Kanagawa Nat Preserv Soc* 13: 1-6 (in Japanese)
- Armstrong PB, Child JS (1965) Stages in the normal development of *Fundulus heteroclitus*. *Biol Bull* 128: 143-168
- Ballard WW (1969) Normal embryonic stages of *Gobius niger* jozo. *Pubbl Staz Zool Napoli* 37: 1-17
- Ballard WW (1973) Normal embryonic stages for salmonid fishes, based on *Salmo gairdneri* Richardson and *Salvelinus fontinalis* (Mitchill). *J Exp Zool* 184: 7-26
- Ballard WW (1986) Stages and rates of normal development in the Holostean fish, *Amia calva*. *J Exp Zool* 238: 337-354
- Ballard WW, Mellinger J, Lechenault H (1993) A series of normal stages for development of *Scyliorhinus canicula*, the lesser spotted dogfish (*Chondrichthyes: Scyliorhinidae*). *J Exp Zool* 267: 318-336
- Iwamatsu T (1994) Stages of normal development in the medaka *Oryzias latipes*. *Zool Sci* 11: 825-839
- Kageyama T (1980) Cellular basis of epiboly of the enveloping layer in the embryo of medaka, *Oryzias latipes*. I. Cell architecture revealed by silver staining method. *Develop Growth Differ* 22: 659-668
- Karlstrom R, Kane DA (1996) A flipbook of zebrafish embryogenesis. *Development* 123: 461
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. *Develop Dynam* 203: 253-310
- Matsui S (1986) Studies on the ecology and propagation of the ice goby, *Leucopsarion petersii* Hilgendorf. *Sci Bull Fac Agr Kyushu Univ* 40: 135-174
- Nakatsuji T, Kitano T, Akiyama N, Nakatsuji N (1997) Ice goby (shiro-uo), *Leucopsarion petersii*, may be a useful material for studying teleostean embryogenesis. *Zool Sci* 14: 443-448
- Westerfield M (1993) The zebrafish book 2nd ed, University of Oregon Press, Eugene, Oregon
- Wourms JP (1972) Developmental biology of annual fishes 1. Stages in the normal development of *Austrofundulus myersi* Dahl. *J Exp Zool* 182: 143-168

(Received March 1, 1999 / Accepted June 5, 1999)