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Authors: Nakatsuji, Takako, Kitano, Tadashi, Akiyama, Nobuhiko, and Nakatsuji, Norio

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Ice Goby (Shiro-uo), *Leucopsarion petersii*, May Be a Useful Material for Studying Teleostean Embryogenesis

Takako Nakatsuji[1], Tadashi Kitano[1], Nobuhiko Akiyama[1] and Norio Nakatsuji[2]*

[1]*Tokai University School of Marine Science and Technology, Shimizu 424, Japan*
[2]*Mammalian Development Laboratory, National Institute of Genetics, Mishima 411, Japan*

ABSTRACT—The highly diverse Perciformes are the most numerous and most advanced group of the teleosts, but experimental embryology has almost completely neglected them. The ice goby (shiro-uo), *Leucopsarion petersii*, deserves attention as a useful species for this purpose. It is readily available commercially from fishermen, and their mature adults can be easily kept and bred in the laboratory. Its totally transparent eggs and embryos develop swiftly and easily under simple culture conditions. Cleavage is finished in 17 hr at 20°C and most organs are established in 3 days. Main stages of embryonic development and methods of handling are described.

INTRODUCTION

Developmental processes of fish embryos have been studied by using various species as experimental materials. Transparency of the embryo has been an important consideration for such studies. For example, transparent *Fundulus heteroclitus* embryos have been used by Trinkaus and others to analyze cell migration during the epiboly and gastrulation (Trinkaus and Erickson, 1983; Trinkaus *et al.*, 1992; Trinkaus, 1993, 1996). Also, the medaka, *Oryzias latipes*, has been a useful material because of easiness of the maintenance and obtaining embryos in the laboratory and presence of many mutant strains (Kageyama, 1977; Iwamatsu, 1994; Ozato and Wakamatsu, 1994), although a relatively thick chorion and large oil droplets in the yolk are sometimes hindering clear observation. These two species belong to the same group of teleost, Cyprinodontiformes. Ballard has been one of those interested in evolutionary and comparative aspects of fish embryogenesis, and he has described the epiboly and other developmental processes of relatively primitive bony fish belonging to Chondrostei (Acipenseriformes, such as sturgeons) (Ballard and Ginsburg, 1980) or Holostei (Amiiformes, such as *Amia calva*) (Ballard, 1986a, b), and also Chondrichthyes, *Scyliorhinus canicula* (Ballard *et al.*, 1993). He also studied groups of Teleostei belonging to Salmoniformes (Clupeiformes, in older schemes) such as *Salmo* species (Ballard, 1973a, b, c), and also Cypriniformes, *Catostomus commersoni* (Ballard, 1982). Recently, zebrafish *Danio rerio* (*Branchydanio rerio*), which also belongs to Cypriniformes, has been widely used as experimental material because of the transparency of embryos and availability of the systematic method for genetical analysis (Kimmel, 1989; Warga and Kimmel, 1990; Westerfield, 1993; Kimmel *et al.*, 1995).

The most advanced and largest group of the teleosts is Perciformes which include very diverse fish, but their embryogenesis has been hardly examined, except a study of the *Gobius niger jozo* by Ballard (1969). The ice goby (shiro-uo in Japanese), *Leucopsarion petersii*, belongs to this Perciformes group. It is one member of gobies and inhabiting along the coast, and they enter the river for spawning in coastal streams (Matsui, 1986). During the spawning season in spring (January along the southern coasts to May along the northern coasts of Japan), they are caught by fishermen in many areas of Japan. Reproduction, spawning and ecology of the ice goby have been studied (Matsui, 1986; Akiyama and Ogasawara, 1994; Akiyama *et al.*, 1995) but chiefly for fishery science publications. So far, a few reports have described brief observation of the development from the eggs to larvae (Matsui, 1986; Akiyama and Kitano, 1995).

The embryos are transparent and easy to culture in the laboratory, and we could examine their development continuously by using time-lapse video-recording with ordinary transmission microscopy. Our description of their development from cleavage through epiboly from such preparations shows

* Corresponding author: Tel. +81-559-81-6829; FAX. +81-559-81-6828.

the usefulness of this unique species for experimental manipulation.

MATERIALS AND METHODS

Animals

Adults of the ice goby (shiro-uo), *Leucopsarion petersii*, which had been ascending the river when caught by fishermen at Anamizu in Ishikawa Prefecture were purchased in May and transported in the oxygenized packages with river water. Temperature was maintained below 20°C during the transportation. The body length of the mature ice goby was approximately 5 cm.

Gonadal maturation and artificial insemination

Each pair of fish was kept in circulating fresh water in the dark inside a plastic petri dish to bring about the gonadal maturation (Akiyama, 1993; Akiyama and Kitano, 1995). Water temperature was kept 14.0-15.0°C. Within 2 to 3 weeks, ovulated females were selected. Ripe eggs, with the diameters of ca. 600 μm, could be gently pressed from the readiest females into sterilized fresh water and mixed with sperm obtained by mincing mature testes in a 1:1 mixture of the sterilized distilled water and the Medium 199 containing 25 mM Hepes buffer (pH 7.4, Gibco) for artificial insemination.

Microscopy

Fertilized eggs and embryos were cultured in plastic petri dishes in the above described diluted Medium 199. Photomicrographs were taken by using an inverted microscope (Olympus IMT-2) with transmission optics. For time-lapse video recording, an inverted microscope (Nikon Diaphoto TMD) with a bright field 10X or 20X lens was used. Images from a video camera (Hamamatsu Photonics C2400-07ER) was recorded in a Laser Videodisc Recorder (Sony LVR-3000N) at the intervals of 1 min or 30 sec. Temperature was kept at 19-21°C during the recording that lasted for a few days. Tracing of the cell migration during the epiboly was carried out by using an image processor (Hamamatsu Photonics Argus 10).

RESULTS

Cleavage and blastoderm formation

The unfertilized eggs after spawning are shown in Fig. 1. These eggs should be fertilized immediately for normal embryogenesis. One feature of the fertilized egg was a prominent blastodisc which was large and high mound shaped on the yolk mass (Fig. 2a). Transparency of the chorion and yolk because of scarce oil droplets made observation of the blastodisc very easy and clear. The first cleavage divided the blastodisc into two blastomeres (Fig. 2b). The second cleavage was at a right angle to the first one, thus forming 4 round blastomeres of the equal size on the yolk surface (Fig. 2c).

Then, the third cleavage furrow appeared horizontally in the 4 blastomeres at the location dividing them into approximately equal sizes (Fig. 3b). The resulting 8 cell stage blastodisc consisted of the lower tier of 4 blastomeres attached to the yolk mass and the upper tier of 4 blastomeres completely separated from the yolk (Fig. 3c). Intervals between these cleavages were 40-50 min. During the following cleavages, the plane of cell division was not easy to see. The blastodisc maintains itself as a high mound during the morula stage, thus forming the distinct blastoderm of the dome-shape (Fig. 3d-f).

Epiboly

After the blastoderm formation, we saw the beginning of epiboly at approximately 17 hr from the fertilization , when the base of the blastoderm transformed from a flat plane into a concave one engulfing the yolk. If we adjusted the focus of a microscope to the margin of the blastoderm on the yolk surface, we clearly saw the advancing front line of the cell layer and formation of the embryonic shield because of the transparency of the yolk (Fig. 4). Tracing of the individual cells in the time-lapse recording showed that the early phase of epiboly

Fig. 1. A micrograph showing unfertilized eggs immediately after spawning. A scale bar indicates 100 μm.

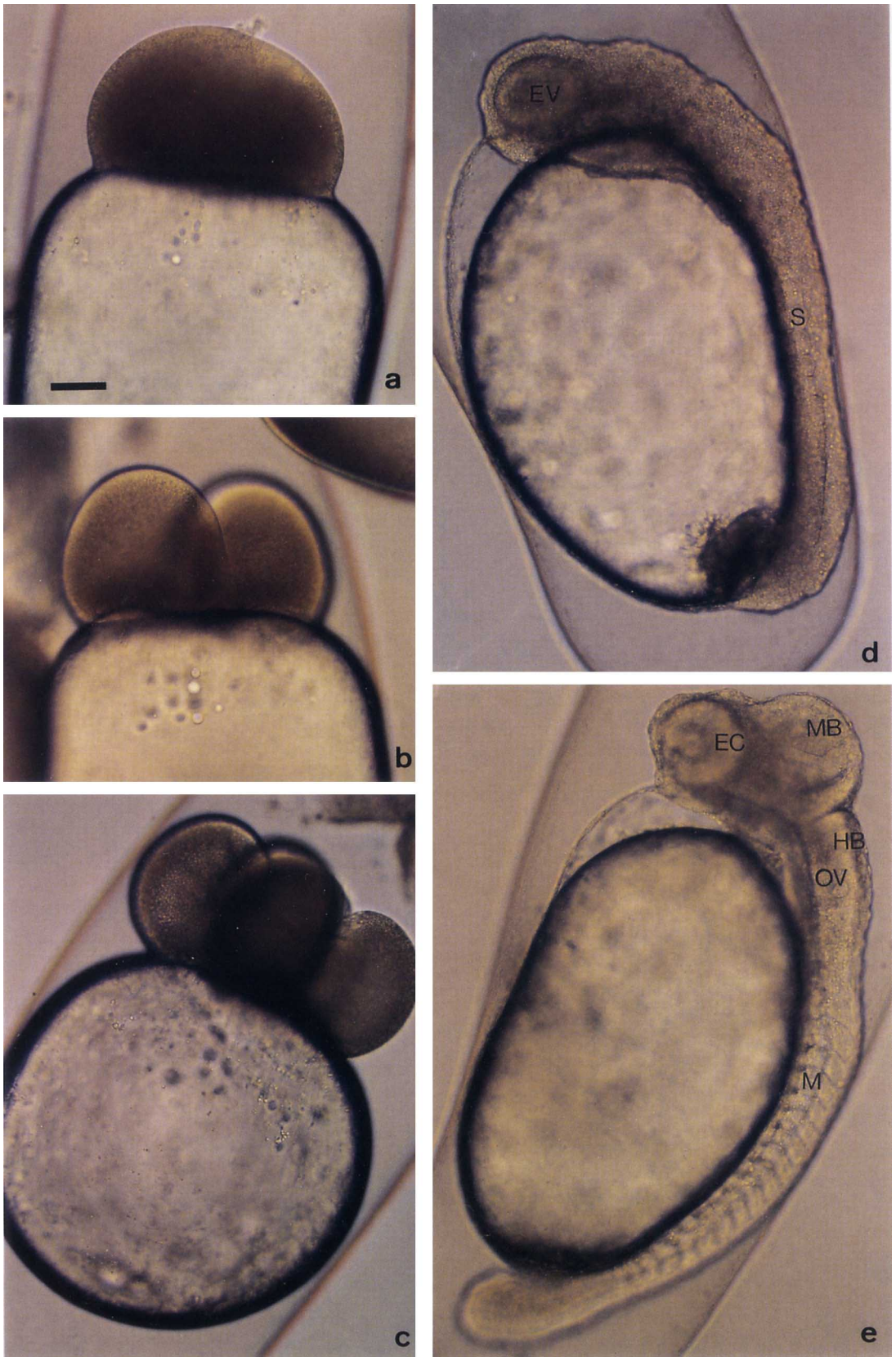

Fig. 2. Ordinary transmission microscopy of the ice goby embryos at 1-cell (**a**), 2-cell (**b**), and 4-cell stage (**c**). Also shown are more advanced embryos after 2 days (**d**) and 3 days (**e**) from the fertilization. EV, eye vesicle; EC, eye cup; HB, hindbrain; M, myotome; MB, midbrain; OV, otic vesicle; S, somites. All photographs are at the same magnification, and a scale bar in (**a**) indicates 100 μm.

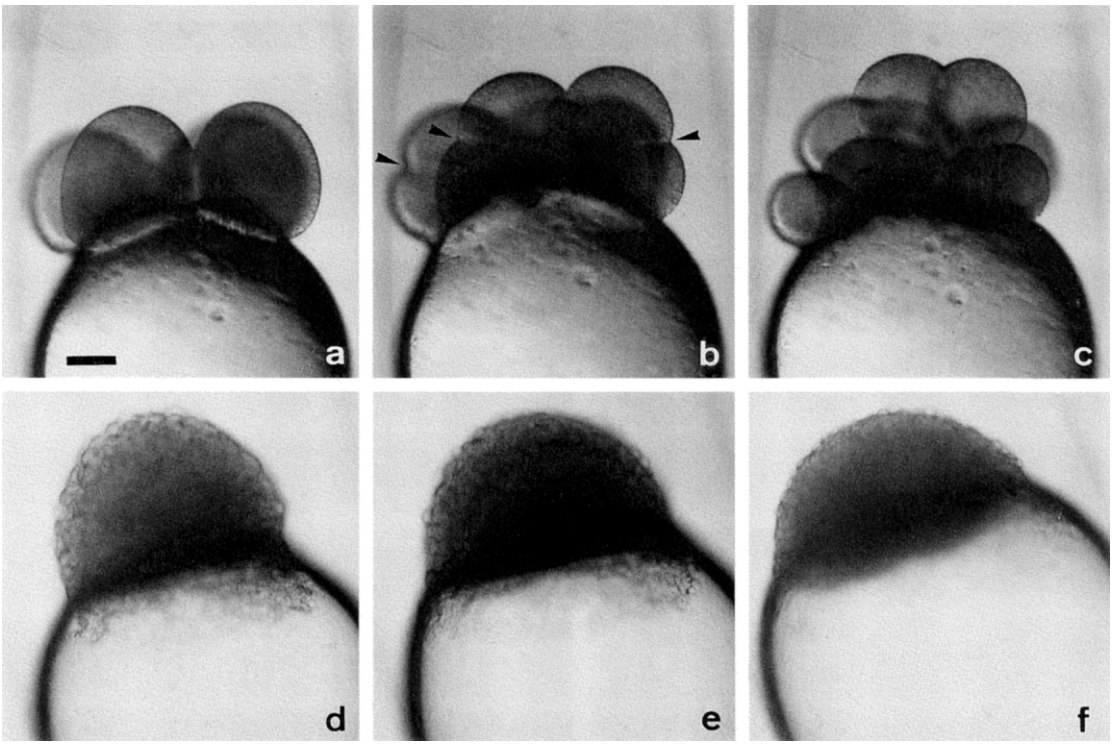

Fig. 3. Photographs printed from the time-lapse video recording. (**a**-**c**) Progress from 4-cell stage (**a**), appearance of furrows of the 3rd cleavage (arrowheads in **b**) and 8-cell stage (**c**). (**b**) and (**c**) are 17 and 28 min after (**a**). (**d**-**f**) Morula stages. Intervals from (**d**); (**e**) 1 hr 0 min, (**f**) 3 hr 1 min. All photographs are at the same magnification, and a scale bar in (**a**) indicates 100 μm.

consisted of relatively uniform translocation of the blastoderm cells toward the advancing margin, and also toward one side of the blastoderm cap where the embryonic shield was being formed (right side in the case of Fig. 4) (Fig. 4d).

During the later phase of epiboly, cells on the blastoderm surface showed translocation toward between the margin and embryonic shield. Also, there were another group of cells, probably deep cells, which showed quite different orientation from the surface cells; they showed rapid migration toward the embryonic shield and away from the epiboly margin (Fig. 4g). It took about 10 hr from the start of epiboly to disappearance of the yolk plug. Again, we could see these morphogenetic events even with the ordinary transmission optics.

Later embryogenesis

Later stages of embryogenesis was also easy to examine because of the transparency of the whole embryo. Also, these embryos could develop normally in a petri dish during the time-lapse recording for 2 or 3 days without any special facilities for the oxygen supply or water circulation. Figure 2d shows a somite-stage embryo with clearly discernible somites and eye vesicles after 2 days from the fertilization. Figure 2e shows a more advanced embryo on the next day with the distinct eye cups, midbrain, hindbrain with otic vesicles, and myotomes.

DISCUSSION

During the initial steps of the embryogenesis, the first two cleavages divides the blastodisc into 4 equal-sized blastomeres on the yolk surface. The third cleavage, however, is horizontal in all blastomeres, and it produces two tiers of blastomeres. Such cleavage pattern is similar to that in the echinoderm and amphibian embryos, but it is different from other fish embryos, in which the early cleavages produce many blastomeres in one layer on the yolk surface. For examples, the first horizontal cleavage occurs between 16-cell and 32-cell stages during the early embryogenesis of the medaka, *Oryzias latipes* (Iwamatsu, 1994), and it occurs still later in the case of zebrafish, *Danio rerio* (*Brachydanio rerio*) (Kimmel *et al.*, 1995). Also, it occurs between 8-cell and 16-cell stages in the primitive Holostean fish, *Amia calva* (Ballard, 1986a). So far, there seems to be no clear report among fish, excluding Agnatha, that the horizontal cleavage occurs as early as the third one. It remains to be examined whether such cleavage pattern is observed in other species of Perciformes.

The present study indicates that the important early morphogenetic movement of the epiboly can be examined in the living embryo of this species. During the early phase of epiboly, the blastoderm cells show the mostly uniform migration. In the later phase, however, there are two distinct groups of cells in the migration patterns. The surface cells show continuation of the epibolic migration. Another cell group, the deep cells, show quite different migration pattern of moving

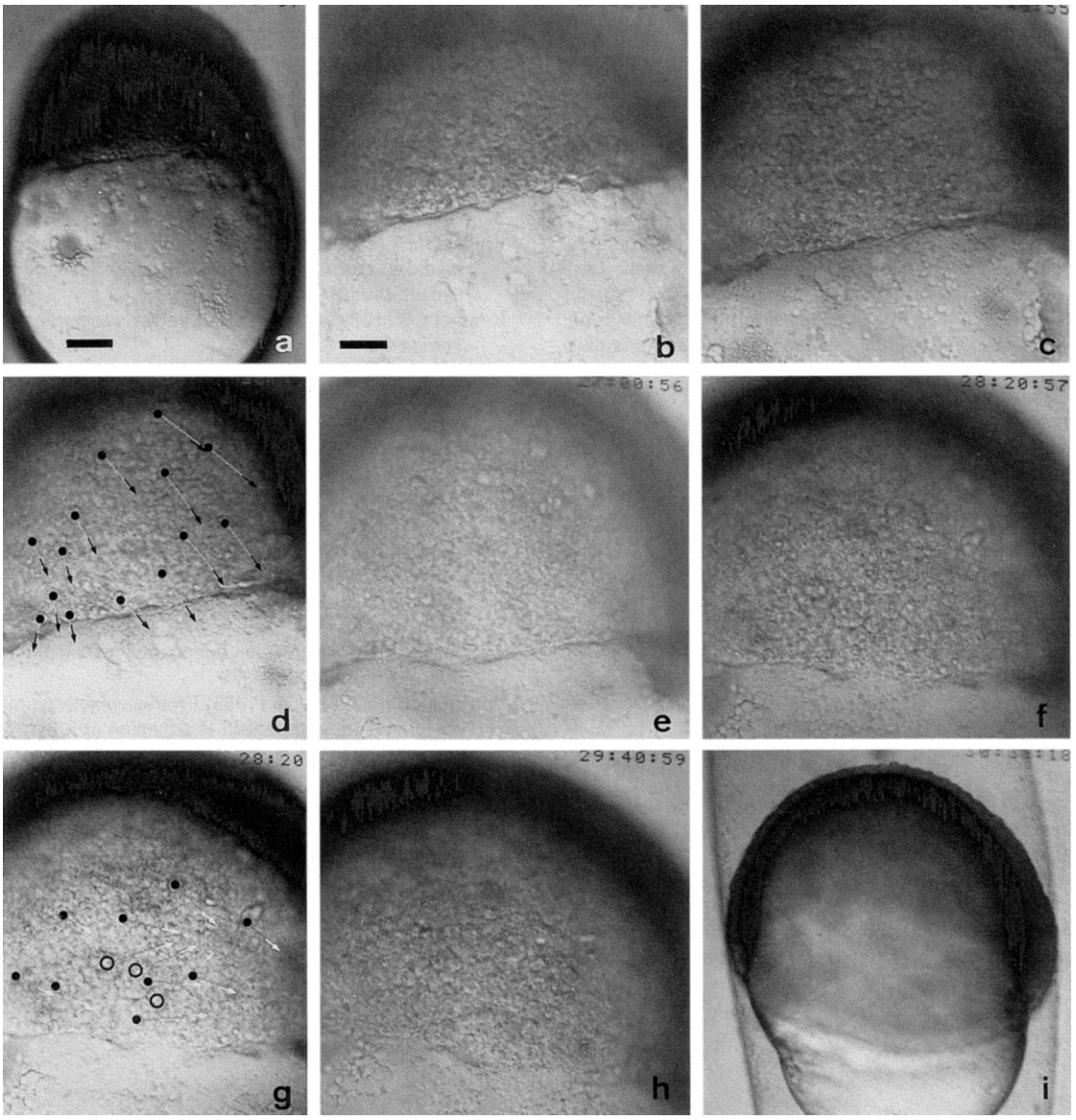

Fig. 4. Progress of the epiboly. Focus of the microscope was adjusted to the advancing margin of the blastoderm layer. (**a**) and (**i**) show lower magnification views of early and late epiboly stages, which correspond to the start and end of the time-lapse series illustrated here. (**b - h**) show advancement of the blastoderm margin at a higher magnification. The embryonic shield was forming on the right side of the illustrated embryo. Intervals from (**b**); (**c**) 1 hr 20 min, (**e**) 2 hr 40 min, (**f**) 4 hr 0 min, (**h**) 5 hr 20 min. Trajectories of individual cells were traced between the stages shown in (**c**) and (**e**) by using an image processor, and illustrated in (**d**) as superimposition of trajectory lines on the embryo image of (**c**). Similar tracing during a later period (**f** to **h**) is shown in (**g**). The start point of each cell is marked by a dot, and the end point after locomotion is marked by an arrow. Three deep cells marked with circles instead of dots in (**g**) showed rapid locomotion away from the blastoderm margin. Scale bars indicate 100 μm (**a**) and 50 μm (**b**), respectively.

toward the embryonic shield and away from the epibolic margin. More detailed analysis of such cell movement and lineage would give important information about embryogenesis in this group of the teleosts.

Beside the transparency of the whole embryo, another advantage of these embryos is that they accomplish normal development in a petri dish without any special facilities to aid gas or water exchange. Also, this species is subject to the coast line fishery in many locations throughout Japan, and it is very unique that the caught fishes are commercially dispatched and distributed as live animals to consumers. Thus, commercial availability of the live adult fishes and effectiveness of the methods to accomplish gonadal maturation and artificial insemination in the laboratory make this species to be potentially a very useful material to study various aspects of fish embryogenesis, especially as an example of the most advanced and prosperous group of the teleost Perciformes. Lastly, there have been attempts to rear the hatched embryos in the laboratory. If it becomes possible to obtain mature fish reared from embryos in the laboratory conditions, this species would be more valuable for research.

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