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Early embryonic development in pikeperch (*Sander lucioperca*) related to micromanipulation

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ABSTRACT: Recently, transplantation of germ cells has attracted attention as a potential technique for efficient reproduction of fish. One of the well-proven techniques to deliver donor germ cells into a recipient is the transplantation of primordial germ cells (PGCs) during the blastula stage. Nevertheless, the application of such techniques so far has been limited to model fish species such as zebrafish, due to the lack of information about early development in many fish species. We propose that pikeperch (*Sander lucioperca*) can be a useful model species for establishing this technique in the order Perciformes, which includes commercially and ecologically important marine species. In this study, we described the important events, namely, embryonic staging, yolk syncytial layer (YSL) formation, and midblastula transition (MBT) during the blastula stage in pikeperch to obtain basic information about early embryonic development. The chorion was softened by treating with 0.2% trypsin and 0.4% urea in Ringer's solution so as to remove it easily by forceps. Although the first cleavage occurred at about 2.5 h post fertilization, blastomeres divided approximately every one hour after this at 15°C. The YSL was formed after the breakdown of marginal cells during the 512- to 1k-cell stage. Cell division analysis by 4'-6-diaminido-2-phenylindole (DAPI) staining revealed that transition from synchronous to asynchronous division occurred after the 10th cleavage (1k-cell stage). Our results indicate that zygotic gene expression (MBT) starts after this stage. Next, we performed blastodisc isolation assay to find the competent stage for embryonic manipulation. Embryos were manipulated by using a microneedle every hour from the 512-cell to the sphere stage, and then developmental rates were evaluated at the hatching stage. The highest survival rate was obtained when we performed this manipulation at the 1k-cell stage. These results clearly showed that the MBT is the best stage for transplantation of PGCs or any cells in pikeperch.

Keywords: blastodisc isolation; blastula; cleavage; early embryogenesis; yolk syncytial layer; midblastula transition

INTRODUCTION

Recent advances in the technology of germ cell transplantation have introduced new avenues of research in fish reproductive management for aquaculture. Transplantation of germline stem cells (GSC) provides an increased supply of donor-derived gametes for surrogate hosts. The fish-seed production is expected to become more efficient by the realization of surrogate production between two different species that have different biological characteristics (Yamaha et al. 2007). For example, the life cycle of the fish might be shortened if a species that has shorter life cycle is used as the surrogate host and produces donor gametes. Moreover, the resources required for fish culture

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(i.e. space, water, food, fuel) might be reduced if a species that has small body size is used as the host for producing gametes of species with a bigger body size. A key factor for implementation of the surrogate production in fish is how to incorporate donor germ cells into the host gonad. Yamaha et al. (2003) transplanted primordial germ cells (PGCs) of crucian carp (*Carassius carassius*) into embryos of the domesticated goldfish (*C. auratus*) and obtained donor-derived gametes from the matured "germline chimeras" (Yamaha et al. 2003). Saito et al. (2011) have shown that a single PGCs transplantation technique works even between marine and freshwater species. Okutsu et al. (2007) have also produced germline chimeras by transplanting rainbow trout (*Oncorhynchus mykiss*) spermatogonia from adult testis into sterile hatched larvae of masu salmon (*O. masou*), and they clearly showed that the germline chimeras generated only gametes of the donor. On the other hand, a transplantation technique has been developed both in tilapia and atherinopsid species, respectively, where spermatogonia from a donor were inserted directly into an adult recipient testis via genital pore (Majhi et al. 2009; Lacerda et al. 2010). However, these aforementioned techniques have also drawbacks (see the review by Robles et al., submitted). To start with the case of PGCs transplantation as shown by Yamaha et al. (2003) and Saito et al. (2011), the manipulation must be performed in a short period during the embryonic stage. In addition, it is difficult to prepare PGCs in the amount appropriate for transplantation. Regarding the spermatogonia transplantation by Okutsu et al. (2007), Majhi et al. (2009) or Lacerda et al. (2010), although numerous cells can be prepared from an adult fish, the efficiency of germline chimerism is low.

The most widely used technique due to its methodological simplicity is blastomere transplantation (BT) (Lin et al. 1992; Wakamatsu et al. 1993; Takeuchi et al. 2001; Yamaha et al. 2001; Nakagawa et al. 2002; Saito et al. 2011). In many fish species, the PGCs localize around the marginal region of the blastodisc during blastula stage (Yoon et al. 1997), and it is difficult to distinguish the PGCs from the somatic blastomeres. Thus, the blastomeres at the marginal region are randomly aspirated into a micro-glass needle and transplanted into a host embryo at the same stage. The confirmation of germline chimerism after BT can be performed by observation of the donor's genetic markers in chimeras' offspring, or by visualization of PGCs with PGCs-labelling molecules as shown by Saito et al. (2011).

The applicability of the BT technique is based on the nature of blastula stage embryo. Blastomeres are pluripotent in their ability to differentiate into any cell types except PGCs, and they last until gastrula. The fate of PGCs has been specified by inheritance of germplasm. Thus, the transplanted blastomeres are capable of contributing to the development of recipient without mismatches of tissue affinity that can cause deformation of the embryo (Saito et al. 2011). In addition, donor-derived PGCs are able to migrate toward the genital ridge of a host embryo at high efficiency after BT (Saito et al. 2011). However, in a series of blastoderm transplantation experiments, the blastomeres lost multipotency and tolerance to surgical manipulation gradually disappeared as the embryo developed (Yamaha et al. 1998).

The loss of blastomere multipotency coincides with mesoderm and endoderm formation. These embryonic layers are initially induced at the equator of the embryo by signals arising from the yolk syncytial layer (YSL) after a midblastular transition (MBT) stage (Mizuno et al. 1996; Chen and Kimelman 2000). Thus, the YSL formation and MBT stages are critical initial steps in embryonic development. From this point of view, the understanding of developmental changes in the blastula stage is important in order to apply the BT technique in the species to be propagated. However, there are restrictions in only some fish species according to detailed information about both BT technique applicability and embryonic development during blastula stage (Yamaha et al. 1998).

Pikeperch, *Sander lucioperca,* is one of the most important freshwater aquaculture species in many European countries such as the Czech Republic, Denmark, Hungary, Romania, Tunisia, Ukraine, the Netherlands, and Poland (FAO 2015). Pikeperch belongs to the order Perciformes, which includes numerous commercially and ecologically important species. In this study, we described the early embryonic development of pikeperch in details during cleavage up to blastula stages. Then, we performed "blastodisc isolation assay" to determine the best period for micromanipulation of BT transplantation during blastula stage. We

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propose that pikeperch is a suitable candidate as a recipient species for applying surrogate production technology in the Perciformes.

MATERIAL AND METHODS

Ethics. All experimental procedures were performed in accordance with the National and Institutional guidelines on animal care and experimentation. This study was approved by the Animal Research Committee of the University of South Bohemia in České Budějovice.

Fish and preparation of embryos. Fish were kept in the aquaculture facility of the Faculty of Fisheries and Protection of Waters, University of South Bohemia in Vodňany, Czech Republic during spawning season from March to April in 2013 and 2014. During handling, the fish were anesthetized with clove oil (0.03 ml/l water) (Dr. Kulich Pharma, s.r.o., Hradec Králové, Czech Republic). Ovulation and spermiation were induced by the injection of human chorionic gonadotropin (Chorulon) (Kristan et al. 2013). Eggs were obtained from two different females, and these were fertilized with sperm from three males in filtered and aerated tap water at 15°C.

Dechorionation of embryos. Fertilized eggs were treated for 5–10 min with 0.2% trypsin and 0.4% urea in Ringer's solution (128mM NaCl, 2.8mM KCl, 1.8mM $CaCl_2$ $2H_2O$) buffered by 10mM TAPS to pH 8.5 to remove the stickiness and soften the chorion (Saito et al. 2011). The chorion was manually removed using fine forceps and the dechorionated zygotes were maintained until the completion of epiboly in Ringer's solution buffered by 10mM HEPES to pH 7.5, containing 1.6% albumin on 1% agar-coated dishes. Embryos with intact chorions were cultured in filtered and aerated tap water in individual plastic Petri dishes (diameter 120 mm). Ringer's solution was replaced every 2 h. After the completion of epiboly, the dechorionated embryos were transferred to a 2nd culture solution (1.8mM $MgCl_2$ and 1.8mM $CaCl_2$) containing 0.01% penicillin and 0.01% streptomycin and incubated at 15°C.

Stage definitions. During cleavage period, embryos incubated at 15°C were observed every 15 min under a stereomicroscope Leica M165FC (Leica Camera AG, Wetzlar, Germany) and photographed by a Leica camera DFC425C. Developmental stages were examined for morphological characteristics such as numbers of blastomeres and a shape of the embryo.

Visualization of nuclei for MBT staging. To plot a ratio of embryos with cells that showed anaphase or metaphase cycle at each time point, nuclei of embryos were visualized and observed. More than twenty embryos were fixed every 15 min until 15.5 h post-fertilization (hpf) with Farmer's fixative (75% ethanol and 25% acetic acid) and kept at 4°C overnight, then replaced with 100% methanol and stored at –20°C until analysis. After dechorionation, blastodiscs were removed from the yolk, and the nuclei were stained with 5% 4'-6-diaminido-2-phenylindole (DAPI) in phosphate-buffered saline (PBS) for 10 min. Then, embryos were washed in PBS and cell divisions in embryos were observed under an inverted fluorescence microscope Olympus IX83 (Olympus, Tokyo, Japan) and photographed with a digital camera C10600 ORCA-R2 (Hamamatsu Photonics, Hamamatsu, Japan).

Blastodisc isolation assay. A blastodisc isolation assay was performed so as to find an optimal stage for embryonic manipulation as described by Yamaha et al. (1998); dechorionated embryos were examined at one-hour intervals from 12 hpf to 17 hpf (512-cell to sphere) (Figure 4A). Then, the developmental rate of each experimental group was evaluated at hatching (Figure 4B). Manipulation was performed in Ringer's solution buffered by 10mM HEPES to pH 7.5 containing 1.6% albumin on 1% agar coated dish. Blastodisc was separated from the yolk mass using a fine glass needle, then replaced in the same position. The manipulated zygotes were incubated in the same dish for 30 min to allow healing of the disrupted cells; zygotes were then placed separately in a 96-well culture dish. At least 18 manipulated zygotes were produced for each stage. During mid-somitogenesis stage, manipulated embryos were transferred into the 2nd culture solution and cultured until hatching. We evaluated survival and malformation rates by observing them under the stereomicroscope.

RESULTS

Early embryonic development in pikeperch. The embryos had a single oil globule within the yolk. The mean diameter of the oil globule was 482.4 µm, and it was about one-half of the yolk diameters (mean: 919.4 µm). The oil globule was located in the upper-central area of the yolk until gastrulation (Figure 1). The animal pole and the

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blastodisc faced downward toward the bottom of the dish after gastrulation but the oil globule, which was not fixed in the yolk mass, moved upward toward the vegetal pole.

The pikeperch embryos have meroblastic discoidal cleavage (Figure 1). The first division occurred around 150 min post fertilization (mpf) at 15°C. Then at approximately one-hour intervals the zygotes cleaved synchronously until the blastula period. The blastomeres formed in a monolayer until the 8-cell stage, and then formed a multilayer from 16-cell stage (Figure 1E–L). Periodic cleavages were observed until around the 512-cell to 1k-cell stage by serial photographs: all blastomeres divided into two daughter cells simultaneously. It was not possible to estimate the number of blastomeres beyond the 1k-cell stage because each blastomere became smaller with each division and the changes in the embryo shape were either slight or indistinguishable until the sphere stage

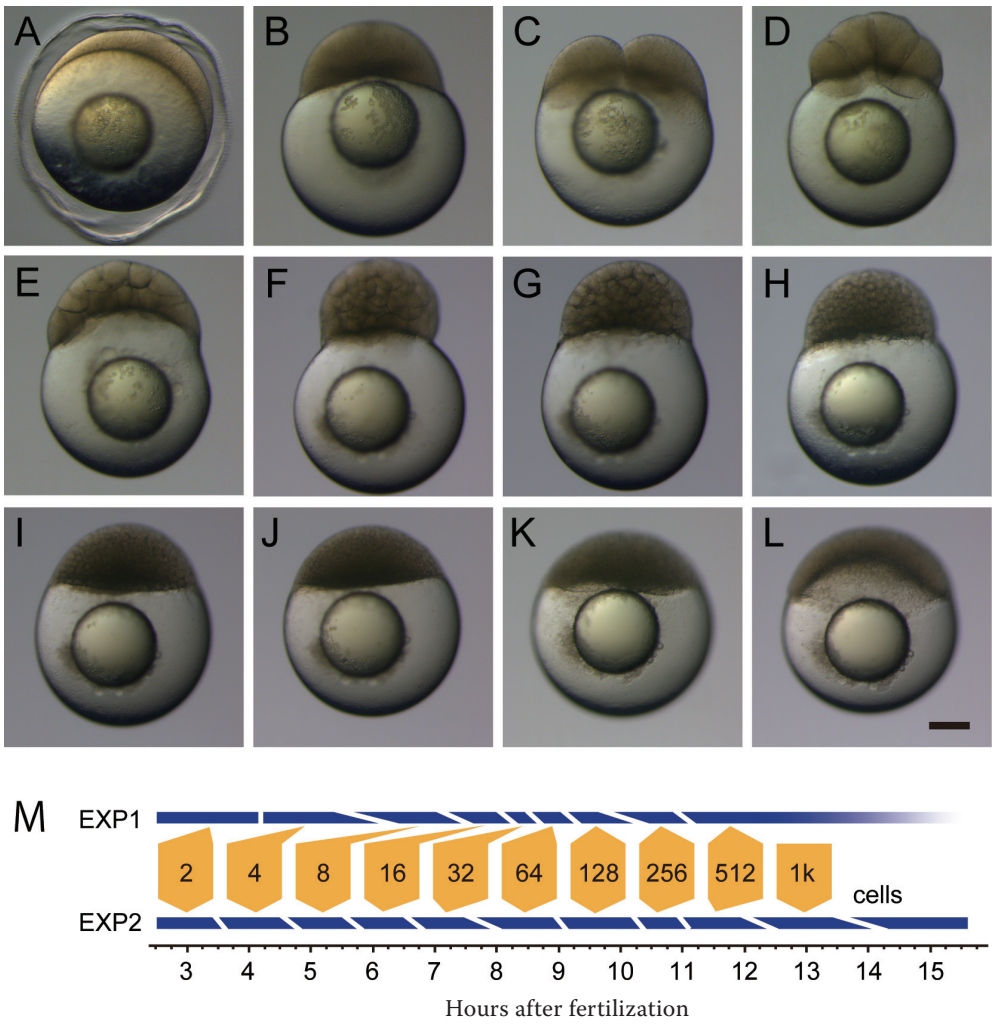

Figure 1. The first 18 hours of pikeperch embryo development

(**A**) 5 min after fertilization; (**B**) 1-cell stage; (**C**) 2-cell stage; (**D**) 8-cell stage; (**E**) 16-cell stage; (**F**) 32-cell stage; (**G**) 128-cell stage; (**H**) 512-cell stage; (**I**) 1k-cell stage; (**J**) 15 hours post fertilization (hpf) blastula; (**K**) 17 hpf blastula (sphere stage); (**L**) dome stage. Note that after the 512-cell stage till the sphere stage (11–17 hpf), the morphological change of embryos is very small. Although embryos become spherical in shape gradually during this period, the change is continuous, and the stage cannot be divided according to the shape; (**M**) developmental speeds varied by batch and embryos. Embryos observed in Experiments 1 and 2 were obtained from different parents in a different season, respectively. 10 embryos were observed for staging in each experiment. White lines on blue bars show the transition of each stage. At the time period of white lines, some embryos showed an advanced stage while some showed a previous stage simultaneously. In the case of Experiment 1, we could not distinguish stages following the 512-cell due to a very slight change in embryo shape. The scale bar indicates 200 μm

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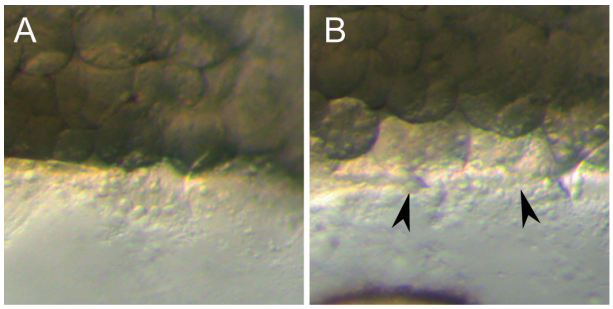

Figure 2. Yolk syncytial layer formation in a pikeperch embryo

(**A**) majority of blastomeres located at the marginal region showed round shape at the 512-cell stage; (**B**) many marginal cells collapsed onto the yolk cell at the 1k-cell stage (arrowheads). The collapsed blastomeres merged into a layer after this stage

(Figure 1I and 1J). The border between blastodisc and yolk was not elevated, and the embryo exhibited a spherical shape at 16.7 hpf (Figure 1K). The embryos became dome-shaped at 18.7 hpf (Figure 1L). It is important to note that there was variation in developmental rate among batches from different parents (Figure 1M). All experiments henceforth were performed using Experiment 2 embryos. During 512-cell to 1k-cell stages, the marginal cells collapsed onto the yolk surface and after this stage, the yolk syncytial layer (YSL) was formed at the marginal zone of the blastodisc (Figure 2).

Mid-blastula transition (MBT). Although there were slight differences in the developing speed among embryos, the majority of the embryos developed synchronously, and the plot graph revealed the general time course of the cell division pattern of the pikeperch embryos (Figure 3A). The time schedule of this synchronous cell cycle corresponded with the observations under the microscope (Figure 3A). The blastomeres divided synchronously up to 1k-cell stage (13.25 hpf) (Figure 3B), and then after this stage (13.5 hpf) the divisions were asynchronous, and cells with the anaphase or metaphase cycle were observed in almost all embryos (Figure 3B). These results clearly showed that the mid-blastula transition started after 1k-cell stage.

Blastodisc isolation assay. In the control group, 95.0% embryos (n = 40) developed and hatched normally. The highest survival rate (73.6%) in the experimental groups was at the 1k-cell stage (n = 19) (Figure 4C), suggesting that this stage is the best for manipulation. Although the survival rates were above 50% in the group of 512-cell (n = 19) and 14 hpf (n = 18), the rates of abnormal development were slightly higher at these phases than

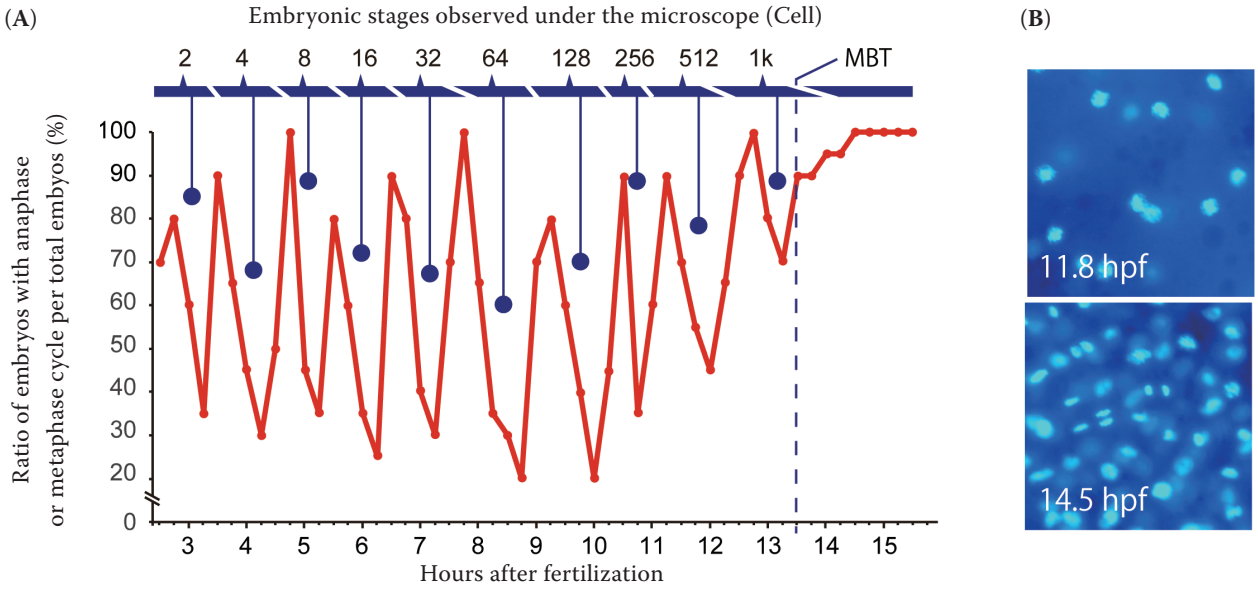

Figure 3. Transition from synchronous cleavage to asynchronous in a pikeperch embryo

(**A**) plots of embryos that have cells in anaphase or metaphase per total embryos showing periodical cycles of cleavage until the 1k-cell stage. After 13.5 h post fertilization (hpf), embryos lost synchronous cell cycles and the mid-blastula transition (MBT) started; (**B**) typical image of synchronous cleavage (above, 11.8 hpf) and asynchronous cell division (below, 14.5 hpf). In the 14.5 hpf embryo, various phases of cell division are observable

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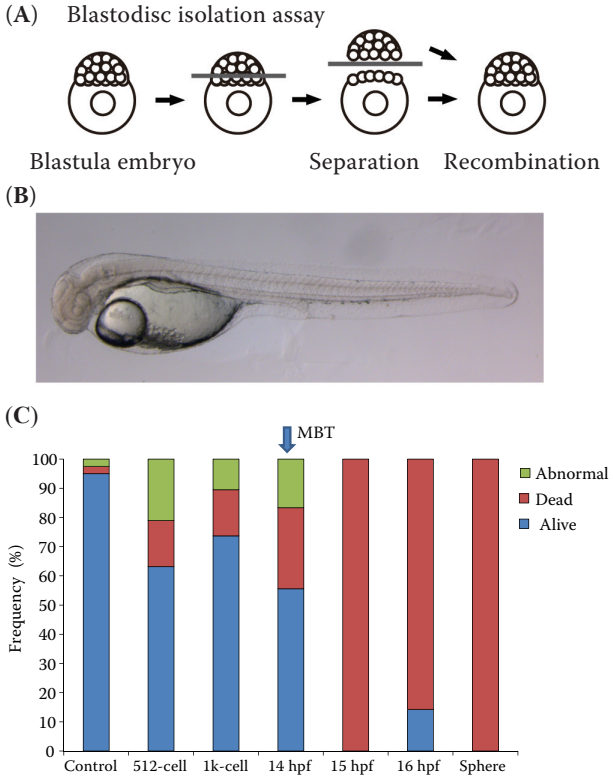

Figure 4. Blastodisc isolation (BI) assay

(**A**) schematic illustration of the BI-assay. Blastodisc was isolated by a micro glass needle and then recombined onto the yolk again. By this assay, we can assess the general reaction against manipulation in each stage; (**B**) hatching stage embryo; (**C**) developmental rate after BI-assay in each stage from 512-cell (11 h post fertilization (hpf)) until 15 hpf was above half the population of the experimental groups. After mid-blastula transition (MBT), some survivals can be detected only at 16 hpf

that of the 1k-cell stage. Almost all manipulated embryos in the 15–17 hpf groups (15 hpf n = 14, 16 hpf n = 14, 17 hpf (sphere) n = 17) showed abnormal shape during development, and they did not survive to hatching.

DISCUSSION

In this study, we described the early embryonic events in pikeperch embryos that encompass the most competent stage for transplantation; this can be considered as basic information for beginning PGCs transplantation in the Perciformes.

The staging of embryonic development in pikeperch has only been described briefly in general terms such as external morphology (Schlumberger and Proteau 1996; Oprea et al. 2014). However, description of the blastula stages during cleavage has not been presented until now in pikeperch. Unfortunately, the staging information obtained for other species based only on the external characteristics cannot be directly applied to pikeperch. In the past two decades, developmental processes during blastulation in fish have been described mainly in cyprinid species, such as zebrafish (Kimmel et al. 1995), loach (Fujimoto et al. 2004), and goldfish (Yamaha et al. 1999; Tsai et al. 2013). Important developmental events during early development can be summarized according to these papers into the following three categories: (1) increasing number of blastomeres by synchronized cell division; during this period, cell number can be counted, (2) yolk syncytial layer (YSL) formation as a preliminary step for germ layer and dorsal axis patterning, and (3) transition from the synchronous to asynchronous cell division, which is known as the mid-blastula transition (MBT). The pattern of germ layer succession can be described using these three transitional phases. We classified these three events as the result of this study as follows:

(1) Synchronized cell division period: 1- to 1k-cell stage. Synchronized cell cycles were repeated 10 times until 13.5 hpf at the culture condition of 15°C. This cycle is identical with that of zebrafish (Kane and Kimmel 1993). However, in goldfish, it is repeated 9 times (Yamaha et al. 1998).

(2) yolk syncytial layer (YSL) formation: In pikeperch, the marginal blastomeres broke down during the 512- to 1k-cell stages and thereafter, nuclei from these blastomeres formed YSL. This sequence is identical to that of zebrafish where the marginal cells undergo a collapse and release their cytoplasm and nuclei by the 512-cell stage (Kimmel and Law 1985). It has been reported in zebrafish and goldfish that the yolk mass with the YSL provides signals that induce the mesoendoderm and the dorsal axis to the pluripotent blastodisc after the mid-blastula stage (Mizuno et al. 1996; Yamaha et al. 1998; Chen and Kimelman 2000). Furthermore, YSL is necessary for epiboly (Kimmel et al. 1995). In this manner, YSL plays a central role in the germ layer patterning and morphogenesis in fishes and is important to understand when this structure is formed during development. The formation of YSL in a pikeperch embryo will be a good marker for staging.

(3) Mid-blastula transition (MBT): A period of transition from synchronous to asynchronous

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cell division is known as MBT. In *Xenopus* and zebrafish, it has been known that zygotic gene expression and active cell migration begin at this stage (Newport and Kirschner 1982; Kane and Kimmel 1993). In pikeperch, asynchronous cell division began after 13.5 hpf (after 1k-cell stage) at 15°C, so this stage is probably the MBT in pikeperch.

In pikeperch embryo, the two events (YSL formation and MBT) occuring around 12–14 hpf related to blastodisc isolation assay demonstrated that embryos have the high competency for manipulation just before ending of blastula period. As a matter of fact, previous studies have shown that the blastomeres remain pluripotent and uncommitted throughout the late blastula and early gastrula stages (Ho and Kimmel 1993; Yamaha et al. 1998). Furthermore, in the present study, embryos had different levels of sensitivity ranges to blastodisc separation comparatively in 512-cell, 1k-cell, and 14 hpf stages. Interestingly, the hatching rate of the 512-cell stage group was slightly lower than that of the 1k-cell stage. At the 512-cell stage, the blastomeres were comparatively larger than those of later stage. It seems that big blastomeres are fragile to manipulation. In fact, blastodisc separation was quite difficult at the 256-cell stage because many blastomeres were destroyed with the glass needle. Thus, it is reasonable to suggest that broken cells affected the development of embryos at the 512-cell stage. On the other hand, embryos after 15 hpf were very sensitive to blastodisc separation, and the developmental rate was quite low. Generally, after MBT the embryo gradually becomes more sphere-shaped, and the tension of the surface of embryos increases as it develops, together with the beginning of the germ layer patterning. This embryonic movement might hinder recovering of a fresh wound after manipulation. Nevertheless, we showed that the optimal stage for micromanipulation in pikeperch embryos is 12–14 hpf at 15°C. This information can be used for PGCs transplantation for pikeperch in the future.

In this study, we described the critical events in early embryonic development and competent stages for micromanipulation in pikeperch embryos. This study suggests that germline chimera can be produced in pikeperch by PGCs transplantation during the blastula stage. The family Percidae includes some important aquaculture species, some are freshwater while others live in seawater. Our results may be applicable to surrogate production techniques in pikeperch such as generation of chimeras between freshwater and seawater species by transplantation of germ cells (Saito et al. 2011). By using pikeperch as a host, many biotechnological applications might be possible in the reproduction of Perciformes fish species.

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