

Morphological differences in embryos of goldfish (*Carassius auratus auratus*) under different incubation temperatures

HIROTARO URUSHIBATA^{*,1,2}, EISUKE TAKAHASHI¹, YU SHIMIZU¹, TOSHIYA MIYAZAKI²,
TAKAFUMI FUJIMOTO¹, KATSUTOSHI ARAI¹ and ETSURO YAMAHA²

¹Nanae Freshwater Station, Field Science Center for Northern Biosphere, Hokkaido University and
²Laboratory of Aquaculture Genetics and Genomics, Faculty and Graduate School of Fisheries Sciences,
Hokkaido University, Hokkaido, Japan

ABSTRACT The goldfish (*Carassius auratus auratus*) is a useful species for embryonic micromanipulations because of its large egg size and wide temperature tolerance. Here, we describe in detail the rate of development and morphological characteristics of goldfish embryos incubated at temperatures between 10°C and 30°C. The cleavage speed increased rapidly as temperature increased. Synchronized cell divisions occurred at 131 min intervals at 10°C, at 33 min intervals at 20°C, and at 19 min intervals at 30°C during the cleavage period. The rate of hatched abnormal embryos significantly increased at temperatures of 26°C and above, while there was no change in the number of abnormal embryos at temperatures less than 24°C. Moreover, the blastomeres around the center of the blastodisc rose in the direction of the animal pole at temperatures less than 14°C. At the lower temperatures, clusters of maternally-supplied germplasm were visualized both at the ends of the first three cleavage furrows and at the border between the lower and upper tiers at the 16- to 32-cell stage, with injection of artificial mRNA and *vasa in situ* hybridization. This study showed that temperature affects not only developmental speed but also the shape of the blastodisc and the distribution of maternally-supplied materials in the blastodisc. By controlling the temperature, it is possible for researchers to prepare many stages of embryos and shapes of the blastodisc from a single batch of eggs.

KEY WORDS: *cleavage, embryonic development, morphogenesis, normal developmental, primordial germ cell, PGC*

Introduction

The familiar ornamental goldfish (*Carassius auratus auratus*) has often been used in experiments of reproductive and developmental biology. For example, bisection at the early cleavage stage of goldfish resulted in the distribution of dorsal maternal factor(s) in the vegetal yolk hemisphere (Mizuno *et al.*, 1997). Graft transplantation showed that at the blastula stage, blastoderm cells are highly pluripotent (Kazama-Wakabayashi *et al.*, 1999, Mizuno *et al.*, 1997), and the yolk syncytial layer has mesoderm-inducing activity (Yamaha *et al.*, 1998). In addition, triploid and gynogenetic diploid embryos are easily induced in goldfish by inhibiting the second polar-body release with heat-shock treatment just after fertilization with normal and irradiated sperm, respectively. Germline chimera has been successfully produced in goldfish by transplantation of blastula graft (Yamaha *et al.*, 2001, Yamaha *et al.*, 2003) and

using primordial germ cells (PGCs) (Goto *et al.*, 2012). These achievements suggest that goldfish eggs and embryos should have characteristics useful for micromanipulation and handling, such as easy artificial ovulation, easy dechoriation, relatively large egg size, and relatively tough chorion, as compared with other experimental model fish, such as the zebrafish *Danio rerio*. It is difficult to slow down developmental speed in zebrafish. Goldfish embryos are also able to survive and develop under a wide range of temperatures. Therefore, the goldfish has the potential to be an excellent host species for studying the interactions between donor germ cells and host somatic cells.

A clear description of each stage of embryonic development is vital to implement embryonic manipulation successfully. The

Abbreviations used in this paper: dpf, days post-fertilization; gfp, green fluorescent protein; hpf, hours post-fertilization; PGC, primordial germ cell.

*Address correspondence to: Hirotaro Urushibata. Laboratory of Aquaculture Genetics and Genomics, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan. E-mail: hirotaro.urushibata@gmail.com -  <https://orcid.org/0000-0002-3546-8434>

Supplementary Material (four figures) for this paper is available at: <https://doi.org/10.1387/ijdb.190144hu>

Submitted: 2 August, 2019; Accepted: 20 November, 2019; Edited by: Makoto Asashima

developmental stages of model animals have been described in great detail especially in zebrafish and medaka *Oryzias latipes* (Iwamatsu, 2004, Kimmel *et al.*, 1995). In goldfish, the developmental stages before gastrulation of embryos incubated at 20°C were defined by Yamaha *et al.*, (1999), and the stages before hatching at 24°C by Tsai *et al.*, (2013). However, as the incubation temperature differed in those two studies, it is difficult to determine the speed of development based on external appearance of the embryos.

Medaka embryos can develop at temperatures ranging from 14 to 34°C. Because the unfertilized eggs of that species can be kept at low temperatures, time-consuming manipulation, such as nuclear transplantation, can be successfully performed with sufficient time (Wakamatsu, 2008). Medaka embryos are also important as educational materials for successive observations of embryogenesis owing to easy preparation of embryos at various stages as induced by different temperatures. Goldfish embryos also develop under a range of temperatures, from 10 to 30°C. However, the developmental staging of goldfish has not been described for incubation under different temperatures.

Here, we describe the developmental stages of goldfish, up to 100% epiboly, under different incubation temperatures, ranging from 10 to 30°C. The stages of embryonic development from epiboly to hatching at 20°C are described in particular detail for comparison with the stages under 24°C as described in Tsai *et al.*, (2013). We observed embryonic development at temperatures from 10 to 30°C. Incubation under lower temperatures affected the early cleavage pattern and distribution of the germplasm. The results are helpful not only in studies of micromanipulation using goldfish embryos before the late blastula stage, but also for making detailed descriptions of the external appearance of embryos at each developmental stage.

Results

Embryonic development

The developmental stages of goldfish embryos incubated at 20°C are summarized in Table 1, and given in comparison with descriptions of their development under 24°C as reported by Tsai

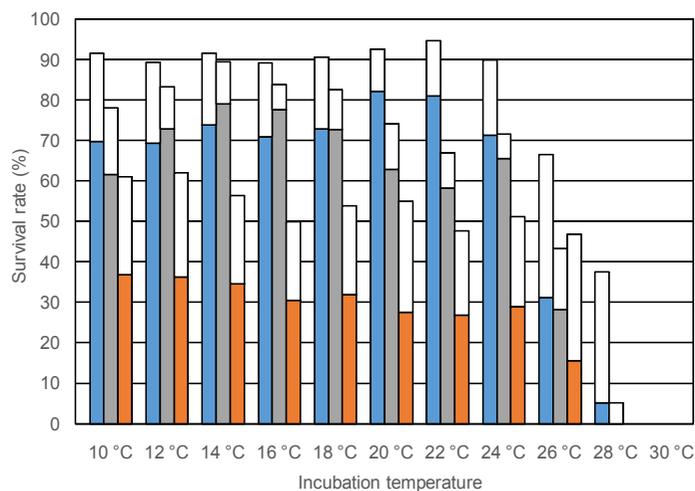
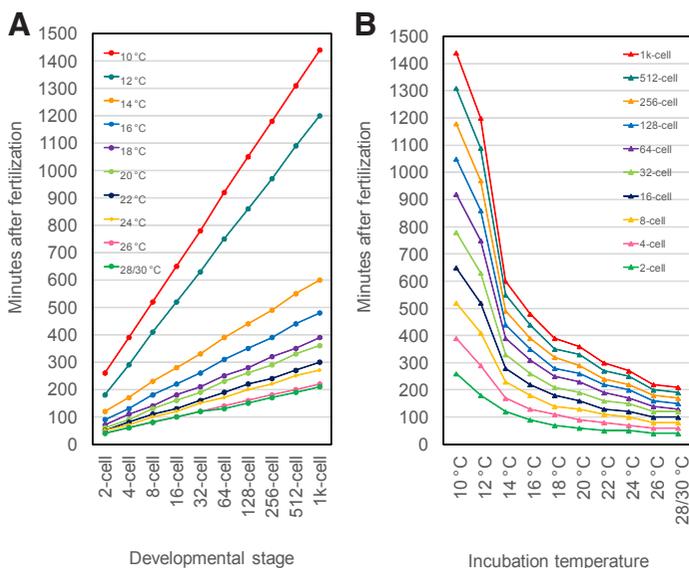


Fig. 2. Survival rate of goldfish embryos incubated at different temperatures. Three batches of embryos (represented by blue, grey, and orange bars) were incubated at each different temperature during the cleavage stage, and then transferred to 20°C; colors indicate normal embryos, and white bars indicate abnormal embryos. Embryos were checked for abnormalities at 5 days post-fertilization.

et al., (2013). As the chorion of goldfish eggs is opaque, dechorionated eggs were used to describe each developmental stage. The embryonic period was subdivided into several stages following Fujimoto *et al.*, (2006). Each developmental stage was named using morphological features in accordance with the stages for zebrafish (Kimmel *et al.*, 1995). Embryonic development of the goldfish was divided into five periods (Table 1): cleavage, blastula, gastrula (Supplementary Fig. S1), segmentation, and hatching (Supplementary Fig. S2). Somite number increased on average by three pairs every 2 h (Supplementary Fig. S3). Hatching in the earliest batch was first observed at 96 hpf, and finished at 138 hpf. Another batch started hatching at the same time, but did not finish until 180 hpf (Supplementary Fig. S4). Fifty percent of the embryos hatched between 114 and 180 hpf. At 20°C, the average hatching time for the three batches was 120 hpf.

Development under different temperatures

Based on embryo morphology under 20°C, the developmental rate to 100% epiboly was determined for different temperatures ranging from 10 to 30°C (Table 2, Fig. 1). Incubated embryos developed rapidly at higher temperatures (>20°C) and slowly at lower temperatures (<20°C). The rate of cell division during early development (before the midblastula transition [MBT]) increased proportionally with increasing temperature (Fig. 1A). Cell division during cleavage was synchronized and occurred at 131 min intervals at 10°C, 33 min intervals at 20°C, and 19 min intervals at 30°C. Between development at the lowest (10°C) and highest (30°C) temperatures, there was a difference of 220 min (about 3.7 h) at the 2-cell stage, 1,230 min (20.5 h) at the 1k-cell stage (i.e. MBT), and 1,690 min (about 28 h) at the late-blastula stage. The time taken from fertilization to each cell division during cleavage

Fig. 1. Rates of development in goldfish embryos incubated at different temperatures. (A) Plot of the time for goldfish embryos to reach each embryonic stage under different cultivation temperatures, and (B) plot of time against temperature for embryos at each stage of development.

decreased exponentially with a change in temperature from 14 to 30°C (Fig. 1B). The time taken for each cell division was relatively long at temperatures of 10–14°C (Fig. 1B).

Frequency of abnormal development at different temperatures

The effect of different temperatures on subsequent development was assessed. Embryos incubated at different temperatures were transferred to 20°C after the 1k-cell stage, and the survival rate and frequency of abnormality, namely body curvature or short body were examined at 5 dpf (Fig. 2). The lowest number of abnormalities was found in embryos incubated at 20°C. As the temperature increased, the number of abnormal embryos at the hatching stage significantly increased. At temperatures of >26°C, more than half of the embryos were abnormal (20°C: 11.2%, 15.2% and 50.0%,

26°C: 53.1%, 34.8% and 66.7%). The frequency of abnormalities did not commensurately increase at temperatures of <20°C, as compared with those kept at >26°C.

External appearance of embryos incubated at lower temperatures

The shape of the blastodisc differed between embryos incubated at the lower and higher temperatures. The blastodisc was round in embryos incubated at the lower temperatures (<14°C), and flat in embryos incubated at the higher temperatures (>14°C) (Fig. 3). The height of the blastodisc decreased with increasing temperatures, whereas the width increased with increasing temperatures (Fig. 4).

Early cleavage patterns were altered in embryos incubated at 10°C. In the 16-cell stage, the fourth cleavage at 10°C differed from

TABLE 1

DEVELOPMENTAL STAGES OF GOLDFISH EMBRYOS AT 20°C

Stage name	Time (20°C) ^a	Tsai <i>et al.</i> , 2013 (24°C) ^b	Characteristics
Cleavage period			
1-cell	40 min	0 min	Formation of the blastodisc on the animal pole
1st cleavage			
2-cell	50 min		Beginning of first cleavage
4-cell	1 h	24 min	The first cleavage furrow
8-cell	1 h 30 min	51 min	The blastodisc formed by 2×2 array blastomeres
16-cell	2 h 10 min	1 h 18 min	Formed by 2×4 array blastomeres
32-cell	2 h 40 min	1 h 45 min	Formed by 4×4 array blastomeres
64-cell	3 h 10 min	2 h 12 min	The fifth horizontal cleavage in the monolayered blastomeres
	3 h 50 min	2 h 39 min	Uncountable blastomeres
Early blastula period			
128-cell	4 h 20 min	3 h 6 min	
256-cell	4 h 50 min	3 h 33 min	
512-cell	5 h 30 min	4 h	
1k-cell	6 h	4 h 27 min	Mid-Blastula transition, E-YSL formation
Late blastula period			
Oblong	8 h	5 h 21 min	I-YSL formation, ellipsoidal shape
Sphere	10 h	5 h 48 min	Spherical shape
Dome	10 h 30 min	6 h 15 min	Beginning of epiboly, bulging
30% epiboly	11 h	6 h 42 min	Progress of epiboly, 30% coverage of a yolk by the blastoderm
Gastrula period			
50% epiboly	12 h	8 h	Half coverage of a yolk cell
Germ ring	13 h	8 h 30 min	Arrest of the epiboly, germ ring
Embryonic shield			
70% epiboly	13 h 30 min	8 h 36 min	Formation of an embryonic shield
90% epiboly	16 h		70% coverage of a yolk cell
90% epiboly	18 h	11 h	90% coverage of a yolk cell, yolk plug
100% epiboly	20 h	12 h	Completion of coverage, tail bud
Segmentation period			
1~3-somite	24 h		EL=2.1 mm, first somatic furrow, optic primordium
4~6-somite	26 h	14 h	EL=2.1 mm, a horizontal crease in the optic primordium, Kupffer's vesicle
7~9-somite	28 h		EL=2.1 mm
10~12-somite	30 h	16 h	EL=2.1 mm
13~15-somite	32 h	20 h	EL=2.1 mm, otic placode
16~18-somite	34 h		EL=2.5 mm, HTA=140°, OVL=0.25 mm, otic vesicle, formation of yolk extension
19~21-somite	36 h		EL=2.9 mm, HTA=140°, OVL=0.25 mm, optic cup and lens placode
30-somite	42 h		EL=3.3 mm, HTA=105°, OVL=0.20 mm, completion of somites, weak heartbeat
Post segmentation period			
2 dpf	48 h		EL=5.0 mm, HTA=105°, OVL=0.15 mm, otolith, median fin fold, early pigment in retina and skin
3 dpf	72 h		EL=5.8 mm, HTA=80°, OVL=0.10 mm, PF (H/W)=1.0, pectoral fin, anus
4 dpf	96 h		EL=7.0 mm, HTA=45°, PF (H/W)=2.0
Hatching period			
5 dpf	120 h		EL=8.1 mm, HTA=30°, PF (H/W)=3.5, hatch

EL, embryo length; HTA, head-trunk angle; OVL, otic vesicle length; PF, pectoral fin; H/W, height/width.

^aHours and minutes after fertilization.

^bHours after fertilization in Tsai *et al.*, (2013) converted to hours and minutes after fertilization.

TABLE 2

DEVELOPMENTAL SPEEDS OF GOLDFISH EMBRYOS UNDER DIFFERENT TEMPERATURE CONDITIONS

Stage	Incubation temperature										
	10 °C (h: min)	12 °C (h: min)	14 °C (h: min)	16 °C (h: min)	18 °C (h: min)	20 °C (h: min)	22 °C (h: min)	24 °C (h: min)	26 °C (h: min)	28 °C (h: min)	30 °C (h: min)
Cleavage speed*	2:11	1:53	0:53	0:43	0:36	0:33	0:28	0:24	0:20	0:19	0:19
2-cell	4:20	3:00	2:00	1:30	1:10	1:00	0:50	0:50	0:40	0:40	0:40
4-cell	6:30	4:50	2:50	2:10	1:50	1:30	1:20	1:10	1:00	1:00	1:00
8-cell	8:40	6:50	3:50	3:00	2:20	2:10	1:50	1:40	1:20	1:20	1:20
16-cell	10:50	8:40	4:40	3:40	3:00	2:40	2:10	2:00	1:40	1:40	1:40
32-cell	13:00	10:30	5:30	4:20	3:30	3:10	2:40	2:30	2:00	2:00	2:00
64-cell	15:20	12:30	6:30	5:10	4:10	3:50	3:10	2:50	2:20	2:10	2:10
128-cell	17:30	14:20	7:20	5:50	4:40	4:20	3:40	3:20	2:40	2:30	2:30
256-cell	19:40	16:10	8:10	6:30	5:20	4:50	4:00	3:40	3:00	2:50	2:50
512-cell	21:50	18:10	9:10	7:20	5:50	5:30	4:30	4:10	3:20	3:10	3:10
1k-cell	24:00	20:00	10:00	8:00	6:30	6:00	5:00	4:30	3:40	3:30	3:30
Oblong	32:00	26:00	14:00	11:00	9:00	8:00	6:30	5:30	4:10	4:00	3:50
Sphere	40:00	32:00	18:00	14:00	11:30	10:00	8:00	6:50	5:20	5:10	4:50
Dome	42:00	33:30	19:00	14:50	12:10	10:30	8:20	7:00	5:40	5:20	5:10
30% epiboly	44:00	35:00	20:00	15:30	12:50	11:00	8:50	7:20	5:50	5:40	5:20
50% epiboly	48:00	38:00	22:00	17:00	14:00	12:00	9:30	8:00	6:30	6:10	5:50
Germ ring	52:00	41:00	24:00	18:30	15:20	13:00	10:20	8:40	7:00	6:40	6:20
Embryonic shield	54:00	42:30	25:00	19:20	15:50	13:30	10:40	9:00	7:20	7:00	6:40
70% epiboly	64:00	50:00	30:00	23:00	19:00	16:00	12:30	10:30	8:40	8:20	8:00
90% epiboly	72:00	56:00	34:00	26:00	21:30	18:00	14:00	11:50	9:50	9:30	9:00
100% epiboly	80:00	62:00	38:00	29:00	24:00	20:00	15:30	13:00	11:00	10:30	10:00

*Cleavage speed means an average interval from 2-cell stage to 1k-cell stage.

that at 20°C. The first latitudinal cleavage appeared sooner, in the 16-cell stage at 10°C as compared with in the 64-cell stage at 20°C (Fig. 5). The blastomeres around the center of the blastodisc rose in the direction of the animal pole and formed a multilayer (Fig. 5). The stage at which the multilayer first appeared was related to temperature (Fig. 6). At 10–14°C, a blastodisc with a multilayer first appeared in the 16-cell stage, while a multilayer first appeared in the 64-cell stage in embryos incubated at 18–20°C. When a single blastomere in the upper layer was injected with fluorescein isothiocyanate (FITC), the dye was maintained in the descendent cells without any diffusion to neighboring blastomeres, indicating that the injected blastomere was isolated (Fig. 7).

Germplasm aggregation pattern at different temperatures

In several fish species, such as ice goby *Leucopsarion petersii* and pond smelt *Hypomesus olidus*, it was reported that germplasm aggregated between the upper and lower tiers of the blastomeres (Saito *et al.*, 2004, Takahashi *et al.*, 2017). To confirm the distribution of the germplasm under cultivation at 10°C, GFP-*buc* mRNA was injected into fertilized embryos at 1-cell stage, thereafter cultivated at 10°C and 20°C, visualized GFP signals in 16-cell embryos were observed in the area around the margin of the blastodisc; however, GFP signals in embryos cultured at 10°C were distributed not only in the marginal area, but also in the boundary between the upper and lower layers (Table 3, Fig. 8). When *in situ* hybridization against *vasa* mRNA was performed, positive signals were detected at both ends of the early cleavage furrows and at the boundary between the upper and lower tiers of the blastomeres (Fig. 8). In many of the embryos kept at 10°C, blastomeres, which were stained with a mottled pattern, were observed with *vasa in situ* hybridization as proof (Fig. 8).

Discussion

Developmental characteristics of goldfish compared with other species

The embryonic development of goldfish under different temperatures was previously described by Tsai *et al.*, (2013). In this study, we described the developmental stages of goldfish from fertilization to hatching, using dechorionated embryos incubated at 20°C. Morphological features, organogenesis, and the head–trunk angle (HTA) (Kimmel *et al.*, 1995) were used to define the different embryonic stages. The sequence of development that we observed did not differ from that reported for goldfish by other authors (Tsai *et al.*, 2013), however the rate of development varied with temperature. The developmental stages described for zebrafish (Kimmel *et al.*, 1995) are similar to those observed in goldfish, although the timing of differentiation, such as the development of otoliths, median-fin folds, and pigmentation, all differed. These differences may relate to numbers of somites and the body proportions of adult fish, although both species belong to the Cyprinidae. Developmental sequences of the loach *Misgurnus anguillicaudatus* (Fujimoto *et al.*, 2006), a species of Cobitidae, differed considerably from the goldfish in regard to the larger numbers of somites.

TABLE 3

THE NUMBER OF GFP SIGNALS IN 16-CELL EMBRYOS AT 10°C INJECTED WITH GFP-BUC mRNA

No. of GFP signals in each embryo (No. of GFP signals in the boundary between the upper and lower layers)	Average
1(0), 1(0), 1(0), 1(1), 1(1), 1(1), 1(1), 2(0), 2(2), 3(0), 3(1), 3(1), 3(1), 3(2), 3(2), 3(3), 4(0), 4(0), 4(1), 4(2)	2.4(0.95)

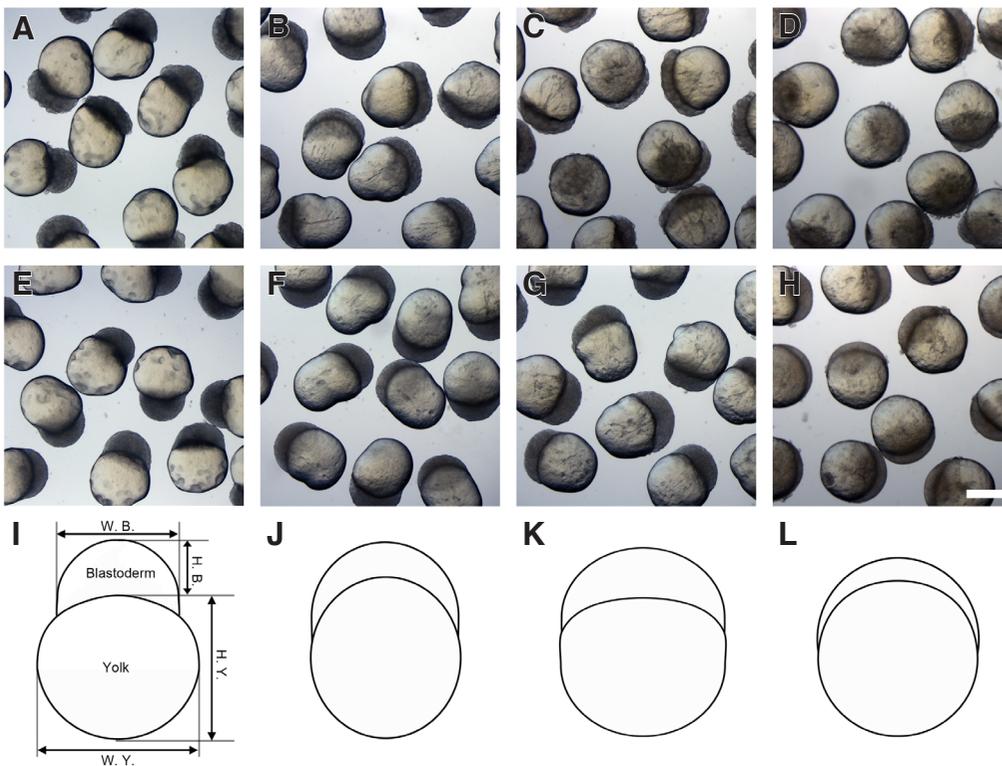


Fig. 3. (A–H) 3D images of blastodisc morphology in goldfish embryos incubated at different temperatures. (A–D) 128-cell stage. (E–H) 1k-cell stage. Embryos in (A) and (E) were incubated at 10°C; (B) and (F) at 20°C; (C) and (G) at 26°C; and (D) and (H) at 30°C. Scale bar = 500 μm. **(I–L)** Schematic illustrations of goldfish embryos at the 1k-cell stage, at representative temperatures. (I) at 10°C; (J) at 20°C; (K) at 26°C; and (L) at 30°C. Abbreviations: W.B., width of the blastodisc along the dorsal–ventral pole axis; H.B., height of the blastodisc along the animal–vegetal pole axis; W.Y., width of the yolk cell along the dorsal–ventral axis, with the yolk diameter measured through the equatorial plane; H.Y., height of the yolk cell along the dorsal–ventral axis, with the yolk diameter measured parallel to the animal–vegetal axis.

Developmental characteristics at different temperatures

The external appearance of goldfish embryos and the timing of the first latitudinal cleavage differed between those incubated at low (<14°C) and high (>14°C) temperatures. Temperature altered the blastodisc height and the cleavage pattern of goldfish embryos in this study. Cytoplasmic structure is supported by microtubules and/or microfilaments that make up the cytoskeleton (Beams *et al.*, 1985). There are many microtubules around the cortex of fertilized eggs in zebrafish. When the cytoskeleton is disrupted, abnormalities are induced in subsequent stages. High

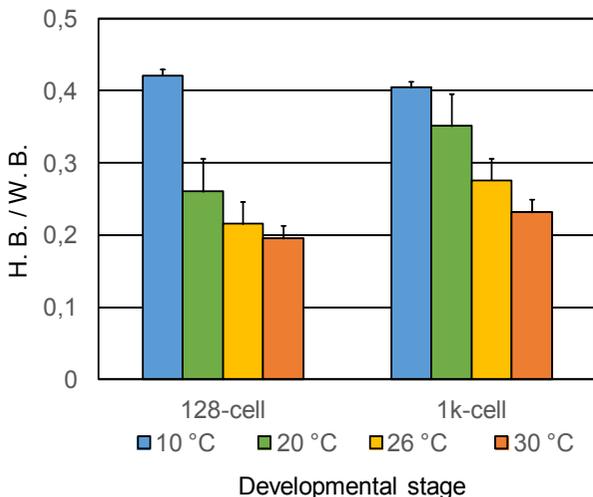


Fig. 4. The ratio that compares H.B. to W.B. in goldfish embryos at 128- and 1k-cell stages at temperatures ranging from 10 to 30°C. H.B., height of the blastodisc along the animal–vegetal-pole axis; W.B., width of the blastodisc along the dorsal–ventral pole axis. Bars denote standard error.

temperatures and high hydrostatic pressure can cause depolymerization of tubulin filaments, affecting dorso-ventral specification, and

resulting in the occurrence of rotational symmetry, microcephaly, a truncated head, or cyclops embryos (Yamaha *et al.*, 2002). In the toad *Xenopus laevis* the microtubular density decreased at a low temperature (Alvarez and Fadic, 1992). In the present study, the time from fertilization to each cleavage stage in goldfish embryos took longer at the lower temperatures (10–14°C). The kinetics of tubulin polymerization in goldfish may change at 16- and 32-cell stage at 10–14°C in contrast to 20°C. Therefore, the morphological changes observed in goldfish embryos may be related to changes in tubulin polymerization as a result of changes in temperature.

In medaka the rate of development was altered with incubation temperature (Iwamatsu, 2004). Medaka embryos survive at temperatures up to 40°C, but development occurs most rapidly at 34°C. The relationship between temperature and incubation period under 34°C for this species is not proportional but exponential. By changing the incubation temperature, developmental speed can be controlled. In goldfish, developmental speeds during the cleavage stage also increased exponentially with increasing incubation temperatures of up to 30°C. There was little difference in the rates of development at 10°C and 12°C.

The longer incubation period at lower temperatures had little effect on the survival rate of fertilized eggs, in contrast with incubation at higher temperatures. Although the external appearance of blastula-stage embryos differed at 10°C and 20°C, these characteristics changed and epiboly could conclude normally.

Germplasm localization

In the present study, GFP signals after injection with GFP-*buc* mRNA and *vasa* signals following *in situ* hybridization were both detected at the boundaries between the lower and upper tiers of blastomeres at the 16- to 32-cell stages in embryos kept at 10°C. In bony fishes, PGCs originated from cells in the maternal cytoplasm, and germplasm aggregated at the site of early cleavage

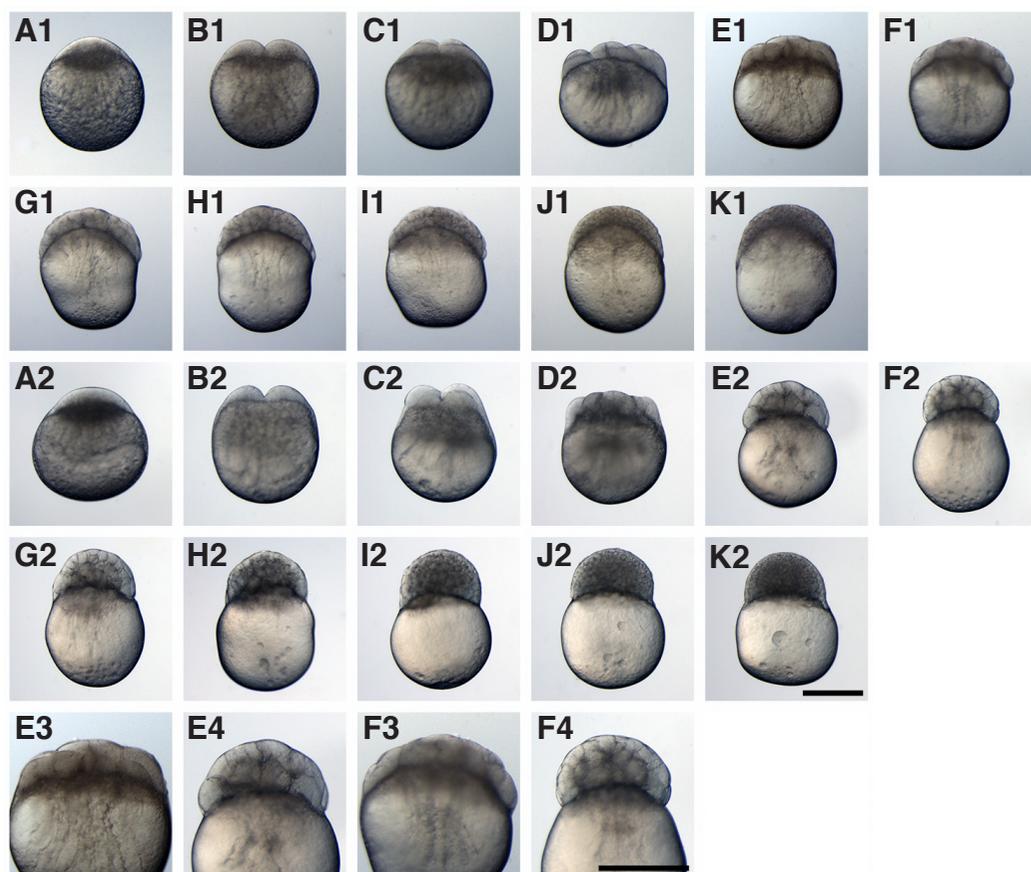


Fig. 5. (A1–K1) 3D images of the cleavage stage of goldfish embryos incubated at 20°C. (A1) 1-cell stage (40 mpf); (B1) 2-cell stage (60 mpf); (C1) 4-cell stage (90 mpf); (D1) 8-cell stage (130 mpf); (E1) 16-cell stage (160 mpf); (F1) 32-cell stage (190 mpf); (G1) 64-cell stage (230 mpf); (H1) 128-cell stage (260 mpf); (I1) 256-cell stage (290 mpf); (J1) 512-cell stage (320 mpf); (K1) 1k-cell stage (360 mpf). (A2–K2) Goldfish embryos incubated at 10°C during the cleavage stage. (A2) 1-cell stage; (B2) 2-cell stage (260 mpf); (C2) 4-cell stage (390 mpf); (D2) 8-cell stage (520 mpf); (E2) 16-cell stage (650 mpf); (F2) 32-cell stage (780 mpf); (G2) 64-cell stage (920 mpf); (H2) 128-cell stage (1050 mpf); (I2) 256-cell stage (1180 mpf); (J2) 512-cell stage (1310 mpf); (K2) 1k-cell stage (1440 mpf, minutes post-fertilization). (E3,F3) High magnification of the blastodisc with a singlelayer. (E3) High magnification of (E1). (F3) High magnification of (F1). (E4,F4) High magnification of the blastodisc with a multilayer. (E4) High magnification of (E2). (F4) High magnification of (F2). Scale bar, 500 μ m.

furrows (Otani *et al.*, 2002, Saito *et al.*, 2004, Yoon *et al.*, 1997). In zebrafish, the germplasm may be visualized by injection with artificial GFP-*buc* mRNA (Bontems *et al.*, 2009). Therefore, the present results show that the germplasm in goldfish aggregates at the boundaries between the lower and upper tiers of blastomeres,

in addition to the normal positions, at the ends of the first three cleavage furrows, under cultivation at lower temperatures. It is reported that germplasm components in zebrafish, which include *dead-end*, *nanos1*, and *vasa* RNAs, are initially present in a wide cortical band at the animal pole and recruited toward the blastodisc periphery by microtubule-dependent movement (Theusch *et al.*, 2006). Low-temperature conditions may affect the movement of germplasm in goldfish embryos. As evidence in this study, mottled staining was observed in the embryos with *vasa in situ* hybridization. This indicated retardation of germplasm movement during the early cleavage stage, though more detailed analysis will be required.

Conclusions

Goldfish (*Carassius auratus auratus*) embryos are useful for embryo manipulation because of their relatively large size and tolerance to a wide temperature range. Developmental speed can be slowed by lowering the temperature, thus enabling researchers to perform complicated and delicate microsurgical operations that require long times. In addition, the round shape of blastoderms in embryos cultivated at lower temperatures enables easy handling for manipulation. Our study shows that the rate of development in goldfish embryos can be changed by altering the temperature, and the embryos are tolerant to such temperature changes. Researchers can prepare embryos of different developmental stages using a single batch of eggs simply by controlling the temperature. In addition, PGCs may increase under low-temperature cultivation, which is useful when employing techniques such as heterochronic

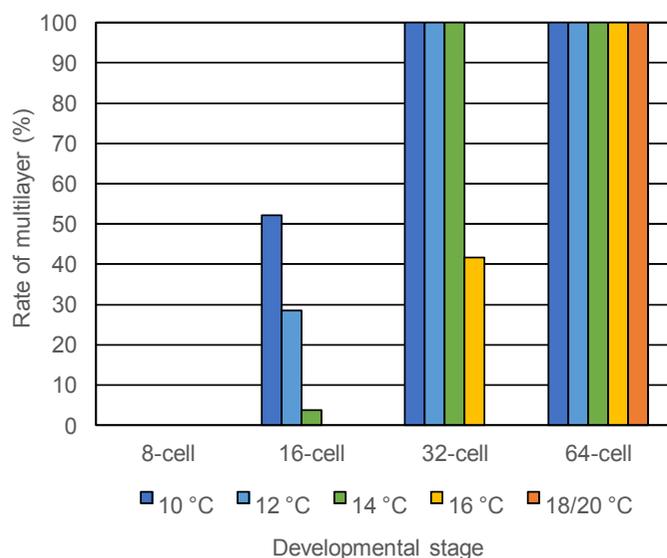


Fig. 6. Percentages of goldfish embryos in which a multilayer formed during the cleavage period, during development at temperatures ranging from 10 to 20°C.

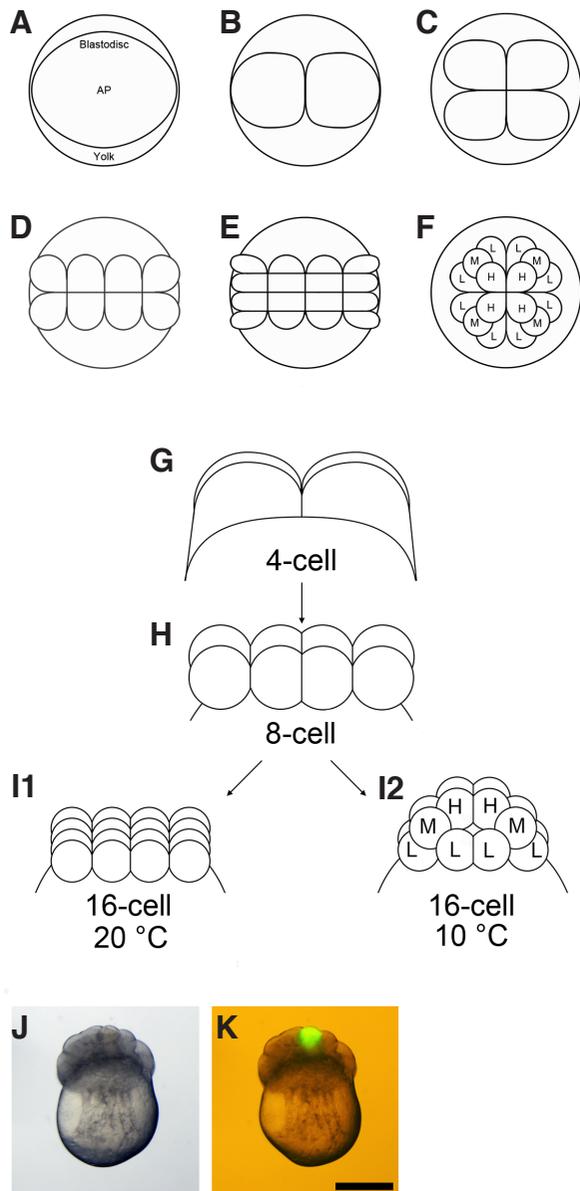


Fig. 7. (Left) Schematic illustrations of the blastomere position in goldfish embryos incubated at 10°C or 20°C. (A–F) Top view of the 1-cell to 16-cell stages. (A) Outer circle denotes the yolk, and inner ellipse the uncleaved blastodisc; AP, animal pole. (B) 2-cell stage; (C) 4-cell stage; (D) 8-cell stage; (E) 16-cell stage, all at 20°C; (F) 16-cell stage at 10°C. (G–I2) Diagrammatic lateral view of the animal pole, showing morphological changes in embryos at the 4-cell to 16-cell stages, when incubated at 10°C or 20°C. (I1) 16-cell stage at 20°C, and (I2) multilayered 16-cell stage at 10°C. H, M and L indicate positions of the blastomeres in the blastoderm, as high, middle or low, respectively. (J, K) 16-cell stage goldfish embryo incubated at 10°C, and injected with FITC solution at this stage; the dye was maintained in the single cell without diffusion. (J) Bright view; (K) fluorescent view. Scale bar, 500 μm.

transplantation and germ-cell transplantation (Saito *et al.*, 2010). Thus, notable embryological phenomena, such as the differentiation potency of blastomeres at early stages, can be studied using goldfish embryos.

Materials and Methods

Ethics

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals in Hokkaido University and Field Science Center for Northern Biosphere, Hokkaido University, Japan (#22-1).

Eggs and sperm

Parent goldfish were kept at the Nanae Freshwater Laboratory at Hokkaido University. Fish were maintained at 10–14°C. Artificial fertilization of goldfish eggs was performed as described by Yamaha *et al.*, (2001).

Dechorionation and incubation conditions

Dechorionated embryos were incubated in 1% agar-coated petri dishes filled with Ringer's culture solution (128 mM NaCl, 2.8 mM KCl, 1.8 mM CaCl₂; pH7), plus 0.01% penicillin, 0.01% streptomycin, and 1.6% albumen.

Intact embryos and dechorionated embryos were divided into 11 groups. Each group of embryos was incubated at a different temperature (10, 12, 14, 16, 18, 20, 22, 24, 26, 28 or 30°C). The intact embryos were observed until the 16-cell stage to determine the exact cleavage speed. The dechorionated embryos were held in 96-well plates and kept at the designated temperature in an incubator until the embryos reached the gastrula period. After gastrulation, the temperature was altered and all embryos were kept at 20°C until the observations were completed. During development, any abnormal embryos were counted and removed.

Measuring the shape of the embryos

The shape of the blastodiscs were measured. At least 10 embryos were randomly selected and the height and width were measured.

Description of developmental stages

The developmental stages of the goldfish were defined using the morphological characteristics described by Tsai *et al.*, (2013). Embryos were observed at 10 min intervals until 24 hours post-

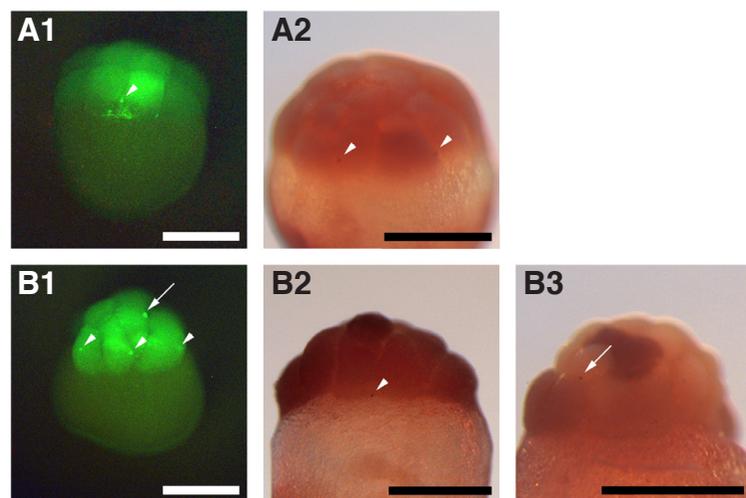


Fig. 8. (Left) Goldfish embryo germplasm at the 16-cell stage. (A) Incubated at 20°C; arrowheads indicate visualized germplasm in the area around the margin of the blastodisc. (B) Incubated at 10°C; arrowheads indicate visualized germplasm in the area around the margin of the blastodisc and arrows indicate those between the upper and lower layers. (A1, B1) Germplasm visualized using green fluorescent protein (GFP) after injection with GFP_{buc} mRNA. (A2, B2, B3) Germplasm visualized with in situ hybridization of vasa probe. Note the blastomeres stained with a mottled pattern in B3. Scale bar, 500 μm.

fertilization (hpf); at 1 h intervals from 24 to 48 hpf; and at 24 h intervals after 48 hpf. At least 20 embryos were randomly selected and observed until epiboly was completed. Following the segmentation period, at least five embryos were randomly selected for observation. After completion of epiboly, the embryonic stages were further described using embryo length (EL), head–trunk angle (HTA), otic vesicle length (OVL), pectoral-fin height (PF), and pectoral-fin height/width (H/W), based on Kimmel *et al.*, (1995), Tsai *et al.*, (2013).

Photographing the external appearance of developing embryos

Embryos without the chorion were incubated in petri dishes with 1% agar, in preparation for photographing. The petri dish containing the embryo was incubated on a heat plate (ThermoPlate, FTP-28190; AS ONE company, Osaka, Japan). The external appearance of each embryo was photographed using a stereoscopic microscope (Leica, MZ16F-RCFL, DFC300FX).

Visualization of a single blastomere

If the 16-cell embryos make multilayered blastoderm, a single blastomere of the 16-cell stage was labeled with fluorescein isothiocyanate conjugated with dextran (FITC-Dextran). Five percent FITC-Dextran (Sigma) solution in 0.2 M KCl was injected into a single blastomere in the animal pole and observed 10 min after the injection.

Analyzing a germplasm localization

Artificially synthesized GFP-*buc* mRNA was injected into the animal or vegetal pole of fertilized eggs to investigate the origin of PGCs, in accordance with the method of Bontems *et al.*, (2009). Capped mRNAs were synthesized *in vitro* using a mMACHINE[®] Kit (Ambion). The artificially synthesized mRNAs were dissolved in 0.2 M KCl at a concentration of 300 mg/ml.

In situ hybridization of *vasa* mRNA was performed to analyze the localization of maternal germplasm, with slight modifications using a 0.4-Kb fragment from the 3'-UTR region of *vas* cDNA in zebrafish as a template, in accordance with the method of Fujimoto *et al.*, (2006). The embryos under incubation at 10°C and 20°C were fixed at the 16- to 32-cell stages with 4% paraformaldehyde for 10 h.

Acknowledgments

The authors are grateful to Shizuo Kimura, Miyuki Kawashima, and members of the Nanae Freshwater Laboratory (Field Science Center for Northern Biosphere, Hokkaido University) for advice and help with the breeding of fish. Cynthia Kulongowski (MSc), with the Edanz Group (www.edanzediting.com/ac), edited a draft of this manuscript. This work was supported by JSPS KAKENHI Grant Numbers 2458163 and 24080099 (to EY).

References

- ALVAREZ, J. and FADIC, R. (1992). Assembly and disassembly of axonal microtubules of the toad *Xenopus laevis* under the effect of temperature. *J Exp Zool* 264: 261-266.
- BEAMS, H.W., KESSEL, R.G., SHIH, C.Y. and TUNG, H.N. (1985). Scanning electron microscope studies on blastodisc formation in the zebrafish, *Brachydanio rerio*. *J. Morphol.* 184: 41-49.
- BONTEMS, F., STEIN, A., MARLOW, F., LYAUTEY, J., GUPTA, T., MULLINS, M.C. and DOSCH, R. (2009). Bucky ball organizes germ plasm assembly in zebrafish. *Curr Biol* 19: 414-422.
- FUJIMOTO, T., KATAOKA, T., SAKAO, S., SAITO, T., YAMAHA, E. and ARAI, K. (2006). Developmental stages and germ cell lineage of the loach (*Misgurnus anguillicaudatus*). *Zool Sci* 23: 977-989.
- GOTO, R., SAITO, T., TAKEDA, T., FUJIMOTO, T., TAKAGI, M., ARAI, K. and YAMAHA, E. (2012). Germ cells are not the primary factor for sexual fate determination in goldfish. *Dev Biol* 370: 98-109.
- IWAMATSU, T. (2004). Stages of normal development in the medaka *Oryzias latipes*. *Mech Dev* 121: 605-618.
- KAZAMA-WAKABAYASHI, M., YAMAHA, E. and YAMAZAKI, F. (1999). The Elimination and Duplication of Lower Part of Blastoderm Effects on the Number of Primordial Germ Cells in Goldfish. *Fisheries Sci.* 65: 577-582.
- KIMMEL, C.B., BALLARD, W.W., KIMMEL, S.R., ULLMANN, B. and SCHILLING, T.F. (1995). Stages of embryonic development of the zebrafish. *Dev Dyn* 203: 253-310.
- MIZUNO, T., YAMAHA, E. and YAMAZAKI, F. (1997). Localized axis determinant in the early cleavage embryo of the goldfish, *Carassius auratus*. *Dev Genes Evol* 206: 389-396.
- OTANI, S., MAEGAWA, S., INOUE, K., ARAI, K. and YAMAHA, E. (2002). The germ cell lineage identified by vas-mRNA during the embryogenesis in goldfish. *Zool Sci* 19: 519-526.
- SAITO, T., GOTO-KAZETO, R., FUJIMOTO, T., KAWAKAMI, Y., ARAI, K. and YAMAHA, E. (2010). Inter-species transplantation and migration of primordial germ cells in cyprinid fish. *Int J Dev Biol* 54: 1481-1486.
- SAITO, T., OTANI, S., FUJIMOTO, T., SUZUKI, T., NAKATSUJI, T., ARAI, K. and YAMAHA, E. (2004). The germ line lineage in ukigori, *Gymnogobius* species (Teleostei: Gobiidae) during embryonic development. *Int J Dev Biol* 48: 1079-1085.
- TAKAHASHI, E., SHIMIZU, Y., URUSHIBATA, H., KAWAKAMI, Y., ARAI, K. and YAMAHA, E. (2017). Migration behavior of PGCs and asymmetrical gonad formation in pond smelt *Hypomesus nipponensis*. *Int J Dev Biol* 61: 397-405.
- THEUSCH, E.V., BROWN, K.J. and PELEGRI, F. (2006). Separate pathways of RNA recruitment lead to the compartmentalization of the zebrafish germ plasm. *Dev Biol* 292: 129-141.
- TSAI, H.Y., CHANG, M., LIU, S.C., ABE, G. and OTA, K.G. (2013). Embryonic development of goldfish (*Carassius auratus*): a model for the study of evolutionary change in developmental mechanisms by artificial selection. *Dev Dyn* 242: 1262-1283.
- WAKAMATSU, Y. (2008). Novel method for the nuclear transfer of adult somatic cells in medaka fish (*Oryzias latipes*): use of diploidized eggs as recipients. *Dev Growth Differ* 50: 427-436.
- YAMAHA, E., KAZAMA-WAKABAYASHI, M., OTANI, S., FUJIMOTO, T. and ARAI, K. (2001). Germ-line chimera by lower-part blastoderm transplantation between diploid goldfish and triploid crucian carp. *Genetica* 111: 227-236.
- YAMAHA, E., MIZUNO, T., HASEBE, Y., TAKEDA, H. and YAMAZAKI, F. (1998). Dorsal specification in blastoderm at the blastula stage in the goldfish, *Carassius auratus*. *Dev Growth Differ* 40: 267-275.
- YAMAHA, E., MIZUNO, T., MATSUSHITA, K. and HASEBE, Y. (1999). Developmental Staging in Goldfish during the Pre-gastrula Stage. *Nippon Suisan Gakkaishi* 65: 709-717.
- YAMAHA, E., MURAKAMI, M., HADA, K., OTANI, S., FUJIMOTO, T., TANAKA, M., SAKAO, S., KIMURA, S., SATO, S. and ARAI, K. (2003). Recovery of fertility in male hybrids of a cross between goldfish and common carp by transplantation of PGC (primordial germ cell)-containing graft. *Genetica* 119: 121-131.
- YAMAHA, E., OTANI, S., MINAMI, A. and ARAI, K. (2002). Dorsal-ventral axis perturbation in goldfish embryos caused by heat and pressure-shock treatments for chromosome set manipulation. *Fisheries Sci.* 68: 313-319.
- YOON, C., KAWAKAMI, K. and HOPKINS, N. (1997). Zebrafish *vasa* homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. *Development* 124: 3157-3165.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Preparation of a fish embryo for micromanipulation: staging of development, removal of the chorion and traceability of PGCs in *Prochilodus lineatus*

Geovanna C.Z. Coelho, Isaac S. Yo, Tatiana M. Mira-López, Paulo S. Monzani, Dilberto R. Arashiro, Takafumi Fujimoto, José A. Senhorini and George S. Yasui

Int. J. Dev. Biol. (2019) 63: 57-65
<https://doi.org/10.1387/ijdb.180348gc>

Inter-species transplantation and migration of primordial germ cells in cyprinid fish

Taiju Saito, Rie Goto-Kazeto, Takafumi Fujimoto, Yutaka Kawakami, Katsutoshi Arai and Etsuro Yamaha

Int. J. Dev. Biol. (2010) 54: 1481-1486
<https://doi.org/10.1387/ijdb.103111ts>

Developmental potential of embryonic cells in a nucleocytoplasmic hybrid formed using a goldfish haploid nucleus and loach egg cytoplasm

Takafumi Fujimoto, Taiju Saito, Suzu Sakao, Katsutoshi Arai and Etsuro Yamaha

Int. J. Dev. Biol. (2010) 54: 827-835
<https://doi.org/10.1387/ijdb.092896tf>

The formation of primordial germ cells from germline cells in spherical embryos derived from the blastodisc of 2-cell embryos in goldfish, *Carassius auratus*

Satoshi Otani, Tomoe Kitauchi, Taiju Saito, Suzu Sakao, Shingo Maegawa, Kunio Inoue, Katsutoshi Arai and Etsuro Yamaha

Int. J. Dev. Biol. (2005) 49: 843-850
<http://www.intjdevbiol.com/web/paper/052027so>

Electrically fused-egg induction and its development in the goldfish, *Carassius auratus*

E Yamaha and F Yamazaki

Int. J. Dev. Biol. (1993) 37: 291-298
<http://www.intjdevbiol.com/web/paper/8398675>

Developmental incompatibility between cell nucleus and cytoplasm as revealed by nuclear transplantation experiments in teleost of different families and orders

S Y Yan, M Tu, H Y Yang, Z G Mao, Z Y Zhao, L J Fu, G S Li, G P Huang, S H Li and G Q Jin

Int. J. Dev. Biol. (1990) 34: 255-266
<http://www.intjdevbiol.com/web/paper/2386727>

Mitochondrial DNA content and mitochondrial gene transcriptional activities in the early development of loach and goldfish

G Wang and S Yan

Int. J. Dev. Biol. (1992) 36: 477-482
<http://www.intjdevbiol.com/web/paper/1338287>

