Title page

Drastic mortality in tetraploid induction results from the elevation of ploidy in masu salmon *Oncorhynchus masou*

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Abstract

Although tetraploidy is considered to be important and useful for the production of sterile triploids and fertile allotetraploids in aquaculture, in most cases the resultant embryos exhibit extremely low survival. In this study, we aimed to clarify the cause of the inviability of tetraploids in masu salmon. Firstly, we compared developmental rates of the first cell cycle among 9 single pairs produced by all possible matings between 3 females and 3 males. The results showed that the fertilized eggs developed almost synchronized among individuals from each pair but asynchronous among pairs and this asynchronous development was more apparent among pairs from different female than those from different male. Secondly, we induced both tetraploidy (4N) and gynogenetic diploidy (G2N) by the same hydrostatic pressure shock (PS) conditions (700 kg/cm^2, 7 min duration) for the first cleavage inhibition using single-pair mating. This experiment was designed to verify whether the cause of mortality of induced tetraploidy was the elevated ploidy itself. Eggs from one female were divided into 2 groups and fertilized with normal or UV-irradiated sperm from one male, respectively. Then each group of eggs was subdivided into 6 groups: one was an intact control and the other 5 groups were treated with PS at every 30 min from 5 to 7 hpf at 10 °C. As a result, the treated eggs of 4N and G2N showed the highest survival (90.8% and 60.6%, respectively) and embryogenesis rate (both 100% relative to surviving eggs) at 33 dpf, only when PS treatment was performed at late prometaphase (6 hpf and 6.5 hpf, respectively). The other PS groups showed extremely low survival (1.5-52.8% and 3.1-14.8%), ceased morphogenetic development and developed into only undifferentiated cell masses. This experiment was repeated 2 more times using other single-pair mating and the results showed the same tendency. The embryonic bodies were confirmed by flow cytometry to have approximately objective ploidy, tetraploid, hypo- or hyper- tetraploid in 4N and diploid or hypo diploid in G2N. However, all tetraploid embryos, even those with normal morphology, began to die

simultaneously around the hatching period (34 dpf), while G2N embryos showed normal morphology and survived beyond 50 dpf (55.6%). Most tetraploid embryos showed malformation and had very poor vascular systems even in normal-looking ones. These results suggest that the mortality of induced tetraploids depends not only on the side effects of the PS treatment but also strongly depends on the induced tetraploidy itself.

Keywords: Masu salmon; *Oncorhynchus masou*; Tetraploidy; Gynogenetic diploidy; First cleavage inhibition; Hydrostatic pressure shock; Mortality; Optimum timing

1. Introduction

Chromosome set manipulation is a technique to control the number and the combination of the haploid set of chromosomes. A large number of technical improvements have been performed to apply it for the enhancement of aquaculture products since the 1980s (Pandian and Koteeswaran, 1998). Inhibition of the first cleavage, as one of the techniques of chromosome duplication, can theoretically induce diploidy in gynogenetically activated eggs and tetraploidy in normal fertilized eggs. Gynogenetic diploidy with completely homozygous genotypes has been successfully induced and then clonal populations have been produced using eggs of these homozygous individuals by the second cycle of gynogenetic development in several finfish species. Although tetraploid fish are expected to be stepping stones to produce sterile triploid and successive auto- and allo- tetraploid individuals by using their diploid gametes, tetraploids are seldom produced due to extremely low survival and frequent occurrence of abnormal development after the treatment. However, successful examples have been reported in a limited number of finfish species, such as rainbow trout *Oncorhynchus mykiss* (Chourrout et al., 1986; Blanc et al., 1987; Diter et al., 1988; Thorgaard et al., 1990), channel catfish *Ictalurus punctatus* (Bidwell et al., 1985; Goudie et al., 1995), African catfish *Clarias gariepinus* (Varadi et al., 1999), tilapia *Oreochromis aureus* (Don and Avtalion, 1988), mud loach *Misgurnus mizolepis* (Nam et al., 2004), common carp (Cherfas et al., 1993), Indian major carp *Labeo rohita* (Reddy et el., 1990) and tench *Tinca tinca* (Flajshans et al., 1993).

In masu salmon, *Oncorhynchus masou*, tetraploidization by inhibition of the first cleavage has also been tried, but viable and normal tetraploid individuals with near-adult sizes have not been obtained yet (Yamazaki and Goodier, 1993; Sakao et al., 2003). Various kinds of developmental abnormalities, such as aberrant cell division, malformations such as nanism in head formation and curved bodies, unexpected aneuploidy and so on, appeared after the treatment (Yamazaki and Goodier, 1993; Sakao et al., 2003). It is unknown whether the

abnormalities are due to side effects of the treatment carried out at inappropriate timing or not. In general, many eggs from multiple females are treated at the same time for tetraploidization in order to maximize survival rates after the treatment. In these heterogeneous eggs, a large number of eggs will be treated at non-optimum timing for duplication and will die due to the induction of abnormalities. While very few embryos will be treated at the optimum timing and be able to develop normally. However, no survivors have been obtained in previous experiment (Sakao et al., 2003). Therefore, tetraploidy, i.e. the elevation of ploidy itself might be a critical factor for inviability in masu salmon.

In the present study, we aimed to clarity the cause of the inviability in induced tetraploid masu salmon. Firstly, we found that the cytological stages from the 1- to 2-cell stage were almost synchronized among individuals from each single-pair mating but asynchronous among groups with different genetic components even when reared at the same water temperature. Based on the above results, secondly, we induced both tetraploidy and gynogenetic diploidy by the same hydrostatic pressure shock conditions using fertilized eggs of single-pair matings to eliminate the influence of genetic component, and examined whether the cause of mortality in induced tetraploids was the elevation of ploidy itself.

2. Materials and methods

Gamete collection

Eggs and sperm were collected from 4 females (♀1-4) and 4 males (♂1-4) in 2002 and 2 females (♀5, 6) and 2 males (♂5, 6) in 2004. Parental fish were all 2-year-old masu salmon and reared at the Mori Research Branch, Hokkaido Fish Hatchery. Eggs and sperm were cooled on ice and transported to the Nanae Freshwater Laboratory, Hokkaido University. Artificial fertilization was performed by the dry method. In this study, all experimental groups were produced by single-pair (one female and one male) mating. The fertilized eggs were

kept in a water bath regulated at 10.0 °C until further treatment.

Cytological staging in single-pair matings

In order to confirm how much differences were in the developmental rates among individuals, at first, eggs from each of 3 females (♀1, ♀2, ♀3) and sperm from each of 3 males (♂1, ♂2, ♂3) were divided into 3 groups, respectively, and then all possible matings were performed. Consequently 9 groups of single-pair matings were obtained. Fertilized eggs were incubated at 10 °C and 15-20 eggs in each of 9 groups were fixed with Bouin's solution at every 30 min in the period from 5 to 7 hours post-fertilization (hpf), when the first cell cycle was cytologically observed in masu salmon (Sakao et al., 2003). The blastodiscs of all fixed eggs were manually isolated from their yolk body and embedded in paraffin. Serial sections were cut 8 μm thick perpendicular to the animal-vegetal pole axis of eggs, stained with hematoxylin and eosin and observed cytologically (Fig. 1).

Genetic inactivation of sperm

In order to induce gynogenetic diploidy, collected milt from each of 3 males (♂4, ♂5, ♂6) was diluted about 50 fold with seminal plasma of masu salmon and genetically inactivated by UV-irradiation according to Onozato and Yamaha (1983).

Inhibition of the first cleavage and induction of tetraploidy and gynogenetic diploidy

In order to inhibit the first cleavage, hydrostatic pressure shock (PS) (700 kg/cm^2, 7 min duration) was applied to the fertilized eggs using a French Press apparatus (Ohtake Works Co., Tokyo). Eggs from ♀4 were divided into two groups and each group of eggs was fertilized with normal sperm (tetraploidy inducing group: 4N) or UV-irradiated sperm (gynogenetic diploidy inducing group: G2N) derived from ♂4, respectively. Fertilized eggs from each group were

subdivided into 6 groups; one was an intact control and the others were PS groups. Each of the 5 PS groups was treated with PS at every 30 min in the period from 5 to 7 hpf. These PS treated groups were designated PS 5h, PS 5.5h, PS 6h, PS 6.5h and PS 7h, respectively. Immediately after the treatments, each group of eggs was incubated separately in hatching tanks containing 10 ºC well water. Several eggs fertilized with normal or UV-irradiated sperm were fixed with Bouin's solution just before each PS treatment in order to observe the cytological stage histologically (Fig. 5). Hatching fry of the control and PS treated groups were reared until 50 days post-fertilization (dpf) (*Trial 1*) (Table 1). Moreover, exactly equal experimental procedures were performed in other two trials using the pair of ♀5 and ♂5 (*Trial 2*) (Table 2) and ♀6 and ♂6 (*Trial 3*) (Table 3). In *trial 2* and *Trial 3*, each group of eggs in 4N and G2N was subdivided into 2 groups: one for calculating survival rate and the other for sampling.

Evaluation of treatments

During the incubation period in *Trial 1*, *Trial 2* and *Trial 3*, dead eggs in all groups of 4N and G2N were periodically counted and removed. Survival rates were calculated from the dead egg number relative to the initial egg number (Fig. 2). In *Trial 1*, at 33 dpf, a few days before the hatching period in the diploid control, external appearances of surviving eggs and surviving embryos after the removal of egg membranes were photographed using a digital camera (CAMEDIA C-3030, Olympus, Tokyo) attached to a stereomicroscope (SZX-12, Olympus, Tokyo) (Fig. 3). Then, eggs with embryonic bodies were counted and their frequencies relative to total and surviving egg numbers were calculated (described as ''embryogenesis rate'') (Table 1). The fertilization rate was taken as the frequency of eggs with embryonic bodies relative to total egg number at 33 dpf (*Trial 1*) (Table 1) and that of cleaved eggs relative to total egg number fixed at 12 hpf obtained from the group for sampling in the control group (*Trial 2*, *Trial 3*) (Table 2, Table 3).

Ploidy determination

In *Trial 2* and *Trial 3*, to examine the effects of hydrostatic pressure shock for chromosome doubling, at 21 dpf, some surviving embryos with embryonic bodies in control groups and PS treated groups were sacrificed to determine ploidy using flow cytometry (FCM) (Ploidy Analyzer, Partec, Germany) (Table 2, Table 3). Embryos used for FCM were obtained from the group for sampling in each experiment. The embryonic body was manually removed from the egg membrane and yolk sac. The embryonic body was then minced with 100 μl of A solution for nuclei extraction (High Resolution DNA Kit type T, Partec, Germany). Twenty minutes later, it was filtered through a 50 μm nylon mesh, supplemented with 500 μl of B solution including DAPI (4-6-diamino- 2-phenylindole dihydrochloride) as a staining solution, and left for 10 more minutes. Then, ploidy determination was performed. Ploidy status was determined based on the relative DNA content using diploid control embryos as a standard of diploid DNA content (2C). In *Trial 1*, we couldn't perform the ploidy determination because of the defect of experimental design (fail to set up the group for sampling).

Statistical analysis

Developmental rates among 9 single-pair matings and between diploid controls and haploid controls induced by single-pair mating were compared by Student's *t*-test.

3. Results

Cytological staging

Cytological staging was performed histologically in the 9 groups from 5 to 7 hpf (Fig. 1). Prophase in the first cell cycle was observed at 5 hpf in all groups, and even at 6.5 hpf in those from ♀3. Metaphase was firstly observed at 6 hpf in all groups from ♀1 and ♀2, but at 7 hpf in

the groups from ♀3. Anaphase was firstly observed only in the cross between ♀1 and ♂1 at 6.5 hpf and in most of the eggs from ♀1 and ♀2 at 7 hpf. The cross between ♀1 and ♂1 gave the fastest development while that between ♀3 and ♂2 gave the slowest one, and up to a 1-hour time lag was observed between the two at the initiation of metaphase (Fig. 1).

Fertilization rate and survival rate of 4N and G2N embryos

In the 4N groups of *Trial 1*, *Trial 2* and *Trial 3*, the fertilization rate of the diploid control was 100% (81/81) (Table 1), 77.8% (7/9) (Table 2) and 100% (9/9) (Table 3), respectively. Most control embryos in *Trial 1* and the survivors at 14 dpf in *Trial 2* and *Trial 3* began to hatch from approximately 34 dpf and survived beyond 50 dpf (Fig. 2A, C, E). In PS 6h of *Trial 1* and PS 6.5h of *Trial 3*, the survival rate maintained high percentage even a few days before the hatching period (90.8% at 32 dpf and 72.5% at 30 dpf), but drastically decreased in the next week and finally all embryos died. In PS 6.5h of *Trial 2* and PS 7h of *Trial 3*, embryos died in a similar manner around the hatching period. In PS 5h of *Trial 1*, the survival rate constantly decreased until 50 dpf. In all other PS treated groups, the survival rates decreased abruptly during the periods from about 7 to 24 dpf, and all embryos died before the hatching period (Fig. 2A, C, E).

In the G2N groups of *Trial 1*, *Trial 2* and *Trial 3*, the fertilization rate of the haploid control was 60.9% (28/46) (Table 1), 66.7% (6/9) (Table 2) and 100% (9/9) (Table 3), respectively. In all 3 trials, haploid control embryos began to die from 30 dpf and they never survived beyond 1 week after the hatching period (Fig. 2B, D, F). In PS 6.5h of *Trial 1*, PS7h of *Trial 2* and PS 7h of *Trial 3*, we obtained stable survivors beyond the hatching period at the rate of 52.5%, 19.0% and 34.6%, respectively. In all other PS treated groups, the survival rates decreased abruptly during the periods from about 7 to 24 dpf and no viable embryos appeared, except for a very few embryos in PS 7h of *Trial 1* (Fig. 2B, D, F).

Embryonic development and external appearance of 4N and G2N embryos

In *Trial 1*, the embryonic development and external appearance were investigated at 33 dpf. In the diploid control, embryonic development was observed in all used eggs (81/81) (Table 1) and embryos showed normal morphology and a normal vascular system (Fig. 3A, G, H). In the haploid control, embryonic development was observed at the rate of 60.9% of total and 100% of surviving eggs, and all survivors expressed haploid syndrome such as microcephali, microphthalmus and curved body (Fig. 3D). In PS 5h and PS 5.5h of both 4N and G2N, embryonic bodies were not detected and only undifferentiated cell masses were observed (Table 1, Fig. 3B, E). These cell masses consisted of nucleated and/or anuclear blastomeres with similar sizes (Fig. 4A-D). In some blastodiscs with wide intercellular spaces, small blastomeres had large granules deeply stained with hematoxylin (Fig. 4E, F). In 4N, embryos were frequently observed in PS 6h (90.8%) but seldom in PS 6.5h (10.0%) and PS 7h (0.8%) (Table 1). Some of these embryos showed near-normal body size and had normal eyes, while the others exhibited slightly malformed morphology such as microcephali, microphthalmus and body flexure, and showed congestion of blood (Fig. 3I). All survivors in 4N had very poor vascular systems running through the heart, gill arch, dorsal aorta, abdominal aorta, other forked arteries, the surface of the yolk sac and so on (Fig. 3I, J) and showed a yellow-tinged color (Fig. 3C) compared to the color of diploid surviving eggs (Fig. 3A). In G2N, embryos were frequently obtained in PS 6.5h (60.6%) but seldom in PS 6h (1.5%) and PS 7h (1.6%) (Table 1). The embryos had normal morphology and normal vascular system (Fig. 3F, K, L) like diploid controls (Fig. 3A). In *Trial 2* and *Trial 3* at 21 dpf, the embryogenesis rates were 56.4% (Table 2) and 88.1% (Table 3) relative to total eggs used in the diploid control, and 56.1% (Table 2) and 88.9% (Table 3) in the haploid one, respectively. In PS treated groups of both trials, embryonic development was observed only in PS 6.5h and PS 7h of 4N and PS 7h

of G2N, but not detected in all other PS groups (Table 2, Table 3).

Ploidy status of 4N and G2N embryos

Ploidy determination of surviving embryos at 21 dpf was performed in *Trial 2* and *Trial 3* (Table 2, Table 3). All diploid control embryos in 4N except for 1 hyper diploid (2.1C) in *Trial 3* were flow-cytometrically confirmed as diploid and all haploid control embryos in G2N were similarly confirmed as haploid. In PS treated groups, only embryos obtained from PS 6.5h and PS 7h in 4N were in the tetraploid range (3.8-4.2C) at high rates (71.4-100%), while the all other embryos were hypo tetraploid (3.6-3.8C) or hyper tetraploid (4.3C). In PS 7h of G2N, all the embryos with embryonic bodies were diploid except for 2 hypo diploids (1.9C) in *Trial 3*.

Cytological staging in 4N and G2N

In the above experiments, the optimum timing for 4N induction was considered to be 6 hpf in *Trial 1* or 6.5 hpf in *Trial 2* and *Trial 3*, while that for G2N was 6.5 hpf in *Trial 1* or 7 hpf in *Trial 2* and *Trial 3*. Therefore, the cytological stage was histologically examined in the specimens fixed just before the PS treatments (Fig. 5). In each diploid and haploid control of all 3 trials, cytological stages advanced sequentially through prophase (formation of the pronuclei with spindle pole) (Fig. 5A, F), early prometaphase (conjugation of both male and female pronuclei) (Fig. 5B, G), late prometaphase (formation and aggregation of the chromosomes in the spindle) (Fig. 5C, H), metaphase (alignment of the chromosomes on the equatorial plate) (Fig. 5D, I) and anaphase (disjunction of the chromosomes toward both spindle poles) (Fig. 5E, J). Especially in the haploid control, the characteristic dense chromatin body, an agglomerate of the sperm genome inactivated by UV irradiation, was observed from prometaphase to metaphase and disappeared at anaphase (Fig. 4H, I, J). The cytological stage estimated to correspond to the optimum timing in both 4N and G2N induction was identified as late

prometaphase in the first cleavage. The first mitotic division progressed appropriately synchronously, but late prometaphase was observed about 30 min earlier in the diploid control than in the haploid one and the significant differences were observed between them in all 3 trials.

4. Discussion

Factors affecting the progression of the first cell cycle

The first cell cycle was cytologically compared among the 9 single-pair matings using 3 females and 3 males. It has been reported that the developmental rate was regulated by the maternal effects such as mitochondrial differences (Robison et al., 1999), egg quality and susceptibility to artificial manipulation (Lou and Purdom, 1984a; Johnstone, 1985; Komen et al., 1991). In this study, the fertilized eggs derived from the same female developed almost synchronously compared to those from the same male, therefore it suggested that the developmental rate should be depending on a maternal effect rather than a paternal one. These results were quite different from our previous report, in which several different stages of the first cell cycle were cytologically observed in the eggs fixed at the same time due to use the pooled gametes and we couldn't determine the optimum timing for tetraploidization (Sakao et al., 2003).

Also in the present study, the sequential mitotic cycles were observed to be delayed by about 30 min in the haploid control. A similar delay in the gynogenetic haploid has been already reported in amago salmon (Kobayashi, 1997), rainbow trout (Kobayashi, 1998) and Pacific oyster (Li et al., 2000) using the eggs from single-pair matings, and therefore it is thought that the cause of the delay was not linked to differences of the parental fish and/or egg quality. In the gynogenetic haploids, the dense chromatin body, which has been observed in other species (Oshiro, 1987; Fujioka, 1993; Kobayashi, 1997, 1998), was observed, but it has

not been clarified yet whether these cytoplasmic characteristics of haploid embryos are related to the delay.

The optimum timing for the first cleavage inhibition

In each 3 trial involving single-pair mating, eggs of the diploid control in 4N and the haploid control in G2N are predicted to develop synchronously in the first cell cycle, considering the experimental results of cytological staging described above. Among the 3 trials we performed, in both 4N and G2N, *Trial* 1 gave the fastest developmental rate and *Trial 2* and *Trial 3* showed moderate rates, with a delay of about 30 min compared to *Trial 1*. It was thought that these differences were due to genetic variance or other factors due to the same age but different year-class: *Trial 1* in 2002, while *Trial 2* and *Trial 3* in 2004. However, in the results obtained from all 3 trials, the treated eggs showed the highest survival rates and embryogenesis rates in both 4N and G2N, only when PS treatment was performed at late prometaphase. It was confirmed in *Trial 2* and *Trial 3* that tetraploidy and diploidy were successfully induced by PS treatment at this stage in 4N and G2N, respectively, though ploidy determination couldn't be performed in *Trial 1* as a matter of experimental design. Additionally, the eggs treated with earlier or later timing than at the optimum late prometaphase exhibited extremely low survival during the first 1 week post-fertilization and ceased morphogenetic development itself, especially in the cases treated at earlier times. In our previous study using pooled gametes, PS treatment was assumed to be effective for tetraploidization during the timing from prometaphase to metaphase (Sakao et al., 2003). Also, other studies concluded that either prometaphase (Nagoya et al., 1990; Kobayashi, 1997, 1998) or metaphase (Purdom et al., 1985; Komen et al., 1991; Nam et al., 1999) was effective for the inhibition of cleavage. However, in this study, more definite differences were observed among the PS treated groups in various experiments due to use single-pair matings, and finally we could conclude that only

late prometaphase was the optimum timing for the first cleavage inhibition in both tetraploidization and gynogenetic diploidization in masu salmon.

The eggs treated at non-optimum timing developed into only undifferentiated cell masses even at the hatching period. It has been reported that such eggs lacking any differentiation of the embryonic body appeared also in the groups treated at non-optimum timing for the tetraploidization (Onozato, 1983; Chourrout, 1984; Yamazaki and Goodier, 1993; Sakao et al., 2003) and androgenic or gynogenetic haploidy (Unger et al., 1998; Lin and Dabrowski, 1998). And these anuclear blastomeres were also observed at the blastula stage in a previous study (Sakao et al., 2003). Anuclear embryos gave rise to arrested development at the blastula stage before the morphogenesis in coho salmon (Aoyagi et al, 1993) and goldfish (Suzuki et al., 1997). The mechanisms of anuclear blastomere formation due to the first cleavage inhibition have not been clarified yet. Recently, Zhang and Onozato (2004) reported the mechanism of chromosome doubling by the first cleavage inhibition, which the disjunction of duplicated chromosomes and the cell division were prevented after the formation of a monopolar spindle in the second cell cycle. However, we did not examine this point. Therefore, more cytological observations will be required to clarify the progress of cell division after the PS treatment at each cytological stage of the first cleavage.

Mortality due to tetraploidy

In many previous studies, it was also reported that tetraploid embryos were successfully induced by the first cleavage inhibition, but they had no survival potential and died soon after hatching or feeding before growing to adult or near-adult size (Lou and Purdom, 1984b; Myers, 1986; Linhart et al., 1991; Arai, 1992; Malison et al., 2001; Sakao et al., 2003). These results suggest that the mortality of tetraploid embryos is not a side effect of the chromosome doubling treatment at the first cleavage, but is the result of the elevation of ploidy status itself, from

diploidy to tetraploidy. Salmonid embryos thus appear not to have tolerance for the genomic change from diploidy to tetraploidy. In salmonids, spontaneous reduplication of the genome rarely happens compared with that in other species because of their unique evolutionary status from a tetraploid ancestor (Onozato, 1983). We have no conclusive idea about why all tetraploid embryos die just around the hatching period. One possible cause of tetraploid mortality that has been proposed is that the reduction of the cell surface area corresponding to the elevation of ploidy might cause deleterious effects on the cellular metabolism (Pandian and Koteeswaran, 1998). It is well known that the increase in cellular DNA content resulting from polyploidization causes an enlargement in nuclear and cellular size but a reduction in the cell number in some organs. In the case of induced triploidy, these alterations should affect on the biological and physiological characteristics causing effects such as deterioration of growth (Solar et al., 1984; Benfey and Sutterlin, 1984), slow response to food (Aliah et al., 1990) and reductions of the cell number in each organ (Swarup, 1959; Aliah et al., 1990), erythrocyte number (Sezaki et al., 1983), circulating blood cell concentration (Sadler et al., 2000), the total amount of hemoglobin in the blood (Benfey and Sutterlin, 1984; Graham et al., 1985), oxygen carrying capacity (Graham et al., 1985; Sadler et al., 2000) and cellular metabolism (Szarski, 1976; Virtanen et al., 1990). Moreover, higher oxygen consumption and higher gill irrigation rate were observed after exhaustive exercise in induced triploids (Kobayashi et al., 1995). In this study, we showed that surviving tetraploid embryos exhibited not only an insufficient vascular system but also blood congestion on the surface of the yolk sac. In the near future, it will be necessary to investigate the physiological function of the vascular system in the tetraploid embryos and alevins.

In conclusion, this study demonstrated that tetraploidy can be induced by PS treated at late prometaphase of the first cell cycle, but such induced tetraploidy is destined to be lethal in masu salmon. Therefore, it is nearly impossible to obtain diploid gametes from mature

tetraploid individuals in this species. We regret coming to this conclusion all the more because allotriploids named “Shinshu salmon”, produced by the normal mating between tetraploid female rainbow trout and diploid male brown trout, were recently produced and commercialized in Japan because they possess various valuable characters. Eventually further investigation is required to clarify the relationship between the elevation of the ploidy toward tetraploidy and the mortality biologically and genetically, and to develop a novel technique of tetraploid production.

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Table 1. Cytological stage just before each PS treatment in the period from 5 to 7 hpf, survival rate and embryogenesis rate of control and PS treated embryos in 4N and G2N of *Trial 1* at 33dpf.

Experiment	Total No.	1st cleavage					Total No	No. of surviving eggs (%)	Embryogenesis rate (%) [1]
		P	EPM	LPM	M	A			
4N group									
Diploid control	—	—	—	—	—	—	81	81 (100)	81 (100)
PS 5h	6	3	3	0	0	0	125	66 (52.8)	0 (0)
PS 5.5h	6	3	2	1	0	0	134	15 (11.2)	0 (0)
PS 6h	6	0	0	6	0	0	141	128 (90.8)	128 (90.8)
PS 6.5h	6	0	0	0	6	0	100	12 (12.0)	10 (10.0)
PS 7h	6	0	0	0	0	6	132	2 (1.5)	1 (0.8)
G2N group									
Haploid control	—	—	—	—	—	—	46	28 (60.9)	28 (60.9)
PS 5h	6	2	4	0	0	0	186	19 (10.2)	0 (0)
PS 5.5h	6	1	5	0	0	0	156	10 (6.4)	0 (0)
PS 6h	6	1	5	0	0	0	131	4 (3.1)	2 (1.5)
PS 6.5h	7	0	0	7	0	0	99	60 (60.6)	60 (60.6)
PS 7h	6	0	0	0	6	0	128	19 (14.8)	2 (1.6)

[1]; Frequency of eggs with embryonic body relative to total eggs.

Abbreviations; P = Prophase, EPM = Early prometaphase, LPM = Late prometaphase, M = Metaphase, A = Anaphase.

Table 2. Cytological stage just before each PS treatment in the period from 5 to 7 hpf, fertility, survival rate, embryogenesis rate and ploidy status of control and PS treated embryos in 4N and G2N of *Trial 2* at 21dpf.

Experiment	Total No. [1]	1st cleavage				Fertility (%) [2]	Total No. [3]	No. of surviving eggs (%)	Embryogenesis rate (%) [4]
		P	EPM	LPM	M				
4N group									
Diploid control	—	—	—	—	—	77.8	39	24 (61.5)	22 (56.4)
PS 5h	6	4	2	0	0	—	42	2 (4.8)	0 (0)
PS 5.5h	7	4	3	0	0	—	53	5 (9.4)	0 (0)
PS 6h	7	3	4	0	0	—	69	2 (2.9)	0 (0)
PS 6.5h	6	0	0	6	0	—	61	21 (34.4)	19 (31.1)
PS 7h	7	0	0	3	4	—	48	6 (12.5)	4 (8.3)
G2N group									
Haploid control	—	—	—	—	—	66.7	41	27 (65.9)	23 (56.1)
PS 5h	5	2	3	0	0	—	39	3 (7.7)	0 (0)
PS 5.5h	7	4	3	0	0	—	44	6 (13.6)	0 (0)
PS 6h	7	3	4	0	0	—	46	6 (13.0)	0 (0)
PS 6.5h	8	3	5	0	0	—	62	7 (11.3)	0 (0)
PS 7h	7	0	2	5	0	—	58	24 (41.4)	18 (31.0)

Experiment	Embryo No. for FCM [1]	Ploidy status (%)			
		1n	2n	4n	Hypo 4n
4N group					
Diploid control	10	0	10 (100)	0	0
PS 5h	—	—	—	—	—
PS 5.5h	—	—	—	—	—
PS 6h	—	—	—	—	—
PS 6.5h	11	0	0	10 (90.1)	1 (9.1)
PS 7h	7	0	0	5 (71.4)	2 (28.6)
G2N group					
Haploid control	10	10 (100)	0	0	0
PS 5h	—	—	—	—	—
PS 5.5h	—	—	—	—	—
PS 6h	—	—	—	—	—
PS 6.5h	—	—	—	—	—
PS 7h	9	0	9 (100)	0	0

[1]; Embryos obtained from the group subdivided for sampling. [2]; Frequency of cleaved eggs relative to total eggs fixed at 12 hpf.
[3]; Total egg numbers of the group subdivided for calculating survival rate. [4]; Frequency of eggs with embryonic body relative to total eggs.
Abbreviations; P = Prophase, EPM = Early prometaphase, LPM = Late prometaphase, M = Metaphase.

Table 3. Cytological stage just before each PS treatment in the period from 5 to 7 hpf, fertility, survival rate, embryogenesis rate and ploidy status of control and PS treated embryos in 4N and G2N of *Trial 3* at 21dpf.

Experiment	Total No. [1]	1st cleavage				Fertility (%) [2]	Total No. [3]	No. of surviving eggs (%)	Embryogenesis rate (%) [4]
		P	EPM	LPM	M				
4N group									
Diploid control	—	—	—	—	—	100	42	37 (88.1)	37 (88.1)
PS 5h	8	3	5	0	0	—	49	1 (2.0)	0 (0)
PS 5.5h	9	0	9	0	0	—	52	5 (9.6)	0 (0)
PS 6h	9	0	9	0	0	—	73	9 (12.3)	0 (0)
PS 6.5h	9	0	3	6	0	—	69	50 (72.5)	43 (62.3)
PS 7h	9	0	0	3	6	—	59	25 (42.4)	17 (28.8)
G2N group									
Haploid control	—	—	—	—	—	100	45	41 (91.1)	40 (88.9)
PS 5h	7	5	2	0	0	—	58	3 (5.2)	0 (0)
PS 5.5h	9	3	6	0	0	—	57	2 (3.5)	0 (0)
PS 6h	8	3	5	0	0	—	62	6 (9.7)	0 (0)
PS 6.5h	9	2	7	0	0	—	75	4 (5.3)	0 (0)
PS 7h	9	0	5	4	0	—	81	51 (63.0)	45 (55.6)

Experiment	Embryo No. for FCM [1]	Ploidy status (%)						
		1n	2n	4n	Hypo 2n	Hyper 2n	Hypo 4n	Hyper 4n
4N group								
Diploid control	8	0	7 (87.5)	0	0	1 (12.5)	0	0
PS 5h	—	—	—	—	—	—	—	—
PS 5.5h	—	—	—	—	—	—	—	—
PS 6h	—	—	—	—	—	—	—	—
PS 6.5h	16	0	0	14 (87.5)	0	0	1 (6.3)	1 (6.3)
PS 7h	3	0	0	3 (100)	0	0	0	0
G2N group								
Haploid control	10	10 (100)	0	0	0	0	0	0
PS 5h	—	—	—	—	—	—	—	—
PS 5.5h	—	—	—	—	—	—	—	—
PS 6h	—	—	—	—	—	—	—	—
PS 6.5h	—	—	—	—	—	—	—	—
PS 7h	12	0	10 (83.3)	0	2 (16.7)	0	0	0

[1]; Embryos obtained from the group subdivided for sampling. [2]; Frequency of cleaved eggs relative to total eggs fixed at 12 hpf.
[3]; Total egg numbers of the group subdivided for calculating survival rate. [4]; Frequency of eggs with embryonic body relative to total eggs.
Abbreviations; P = Prophase, EPM = Early prometaphase, LPM = Late prometaphase, M = Metaphase.

Figure legends

Fig. 1.

Frequency of sequenced cytological stages of the first cleavage at every 30 min from 5 to 7 hpf in the 9 single pairs produced by all possible matings between 3 females (♀1, ♀2, ♀3) and 3 males (♂1, ♂2, ♂3). Values on the right size of each column indicate the time of metaphase initiation (mean ± SD). Different letters indicate significant differences ($P<0.05$) among the developmental rates of different pairs. Asterisks (*) indicate values estimated only from the results obtained by 7 hpf and actual values are expected to be even larger. The fertilized eggs derived from the same female developed almost synchronously compared to those from the same male.

Fig. 2.

Survival rates from 0 to 50 dpf in 4N and G2N groups of *Trial 1* (A, B), *Trial 2* (C, D) and *Trial 3* (E, F). Each trial was performed by using the gametes derived from single-pair mating and the same hydrostatic pressure shock conditions (700 kg/cm^2, 7 min duration) for the first cleavage inhibition. G2N embryos survived beyond 50 dpf, but 4N embryos all died around the hatching period even in the group treated at optimum timing.

Fig. 3.

External appearance of surviving eggs (A-F) and surviving embryos after the removal of egg membranes (G-L) at 33 dpf in *Trial 1*. Diploid control embryos were normal in morphology and had a well-developed vascular system (A, G, H). Haploid control embryos showed haploid syndrome (D). In PS 5h of 4N and G2N, embryonic bodies were not detected at all (B and E). In 4N, embryos were frequently observed in

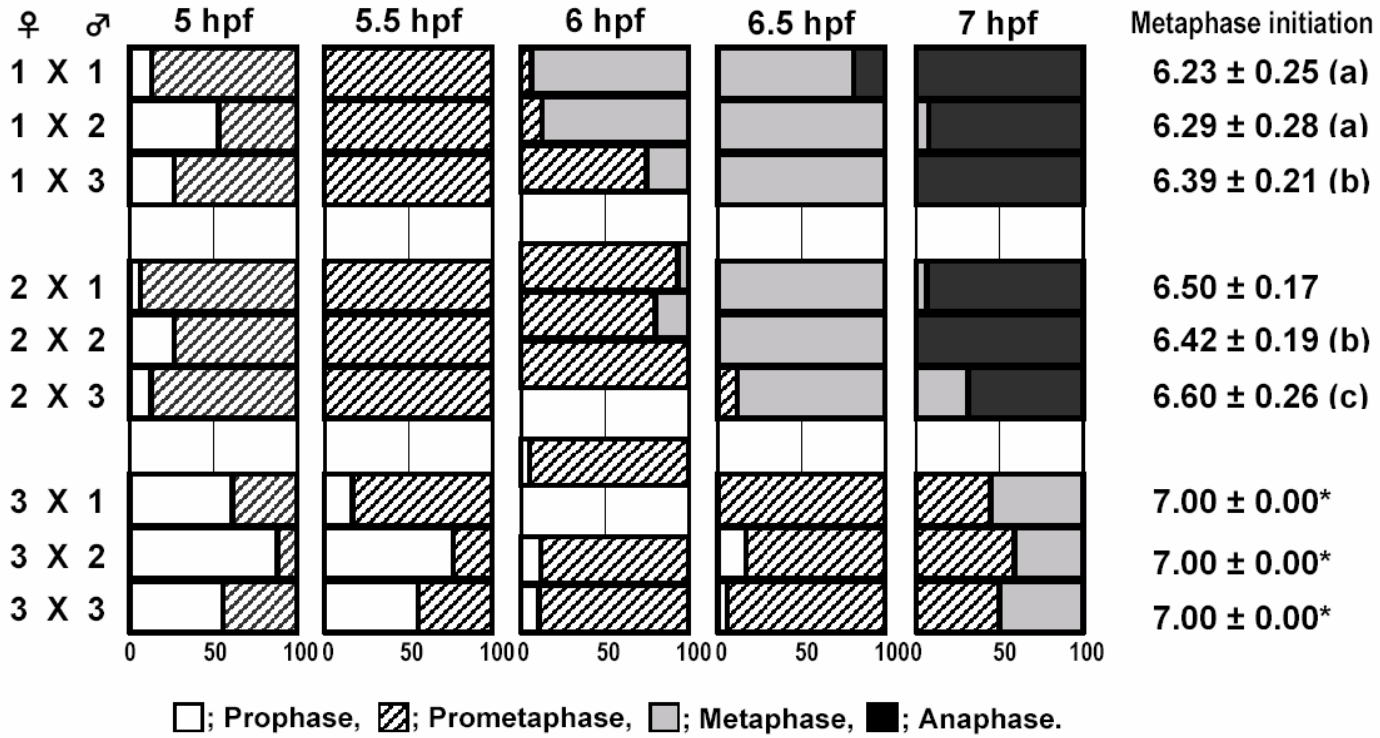
PS 6h (C), but showed body flexure (arrowhead) and had congestion of the blood and a poor vascular system (arrow) (I, J) and showed a yellow-tinged color compared to the color of diploid surviving eggs (A). In G2N, embryos were frequently observed in PS 6.5h (F) and had normal morphology and a normal vascular system (K, L). H, J, L; higher magnification of the head region of G, I and K, respectively. Bar; 5 mm (A-F), 1 mm (G-L).

Fig. 4.

Histological observation of undeveloped PS 5h eggs in 4N of *Trial 1* (Fig. 3B). Sections were cut perpendicular (A, E) or parallel (C) to the animal-vegetal pole axis of eggs. Blastoderms of these eggs consisted of undifferentiated cells, in which nuclei were occasionally observed (arrowheads). In some blastoderms, aggregated cells were stained densely with hematoxylin (arrows). B, D, F; higher magnification of the rectangles in A, C and E, respectively. Bar; 250 μm (A, C, E), 100 μm (B, D, F).

Fig. 5.

Cytological appearance of the first mitotic division of diploid control (A-E) and haploid control (F-J) of masu salmon (*Oncorhynchus masou*) in the period from 5 to 7 hpf. In both the diploid and haploid controls, the cytological stage advanced sequentially through prophase (A, F), early prometaphase (B, G), late prometaphase (C, H), metaphase (D, I) and anaphase (E, J). Only in the haploid control, the characteristic dense chromatin body was observed from prometaphase to metaphase and disappeared at anaphase. pn; pronuclei, sp; spindle pole, ne; nuclear envelope, dcb; dense chromatin body; ch; chromosome, cf; cleavage furrow.


♀ ♂
5 hpf
5.5 hpf
6 hpf
6.5 hpf
7 hpf
Metaphase initiation
1 X 1
1 X 2
1 X 3
2 X 1
2 X 2
2 X 3
3 X 1
3 X 2
3 X 3
6.23 ± 0.25 (a)
6.29 ± 0.28 (a)
6.39 ± 0.21 (b)
6.50 ± 0.17
6.42 ± 0.19 (b)
6.60 ± 0.26 (c)
7.00 ± 0.00*
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□; Prophase, ▨; Prometaphase, ▢; Metaphase, ■; Anaphase.

Fig. 1.

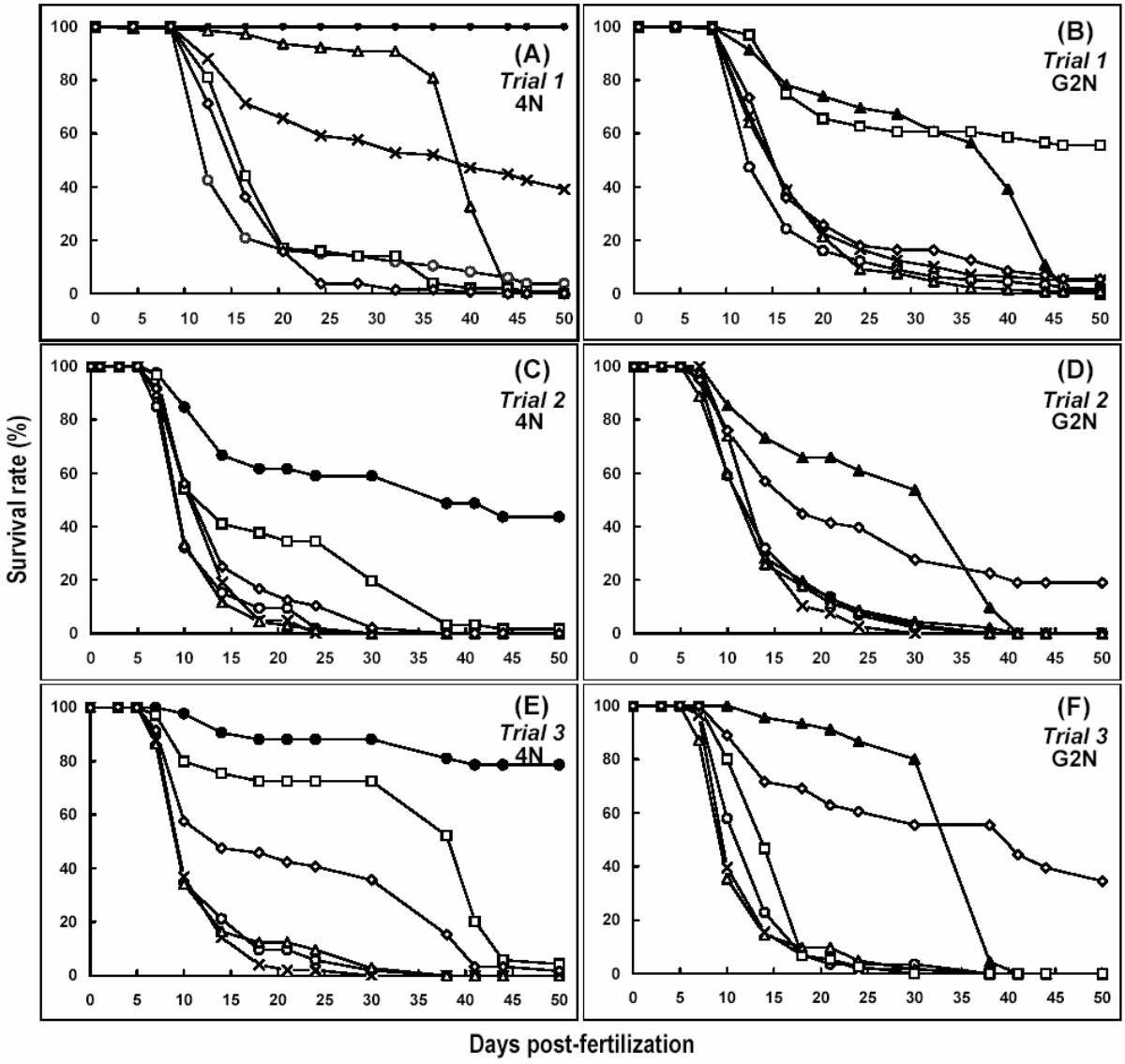
(A)
Trial 1
4N
(B)
Trial 1
G2N
(C)
Trial 2
4N
(D)
Trial 2
G2N
(E)
Trial 3
4N
(F)
Trial 3
G2N
Survival rate (%)
Days post-fertilization
●; Diploid control, ▲; Haploid control, ×; PS 5h, ○; PS 5.5h, △; PS 6h, □; PS 6.5h, ◇; PS 7h.

Fig. 2.

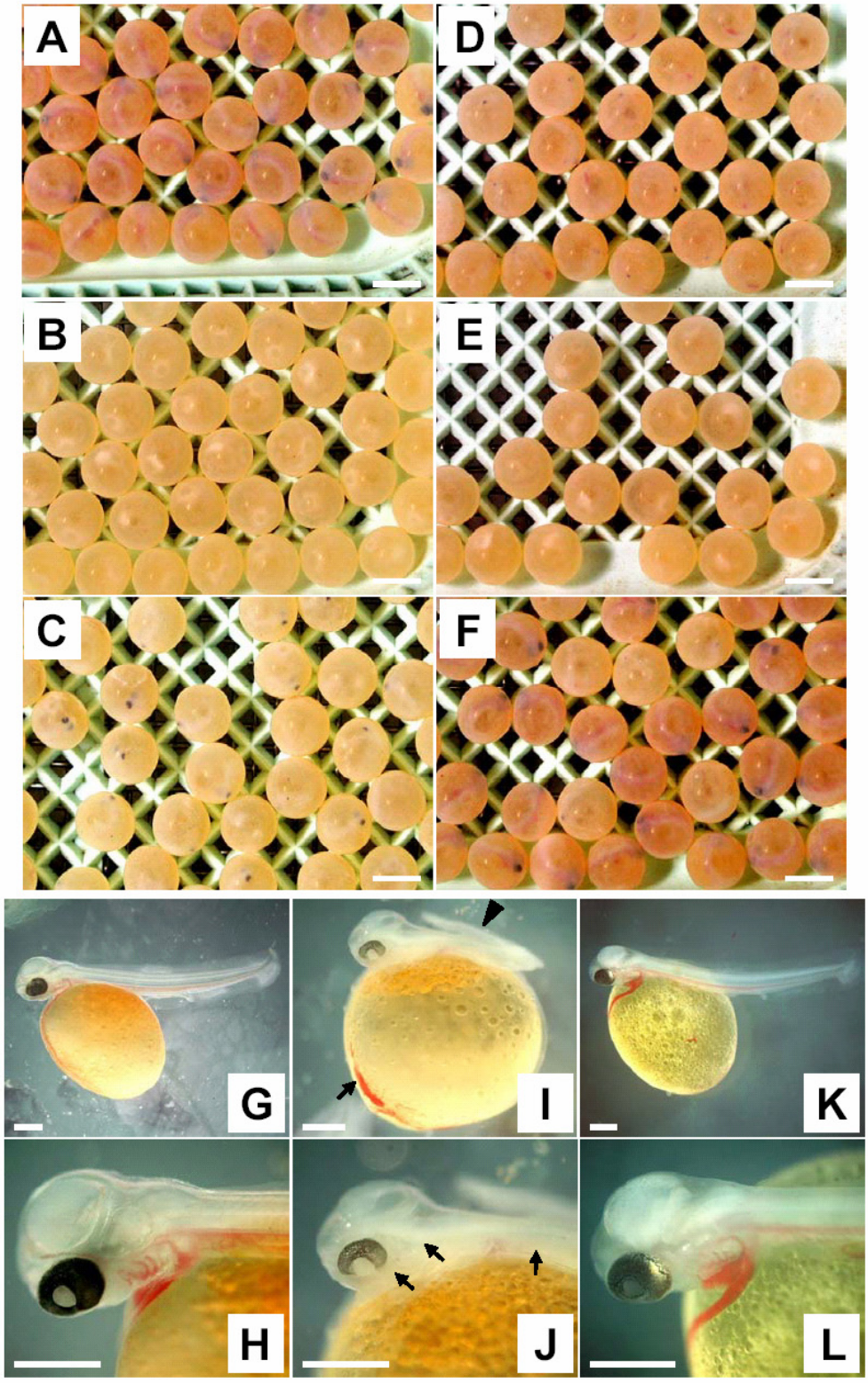

Fig. 3.

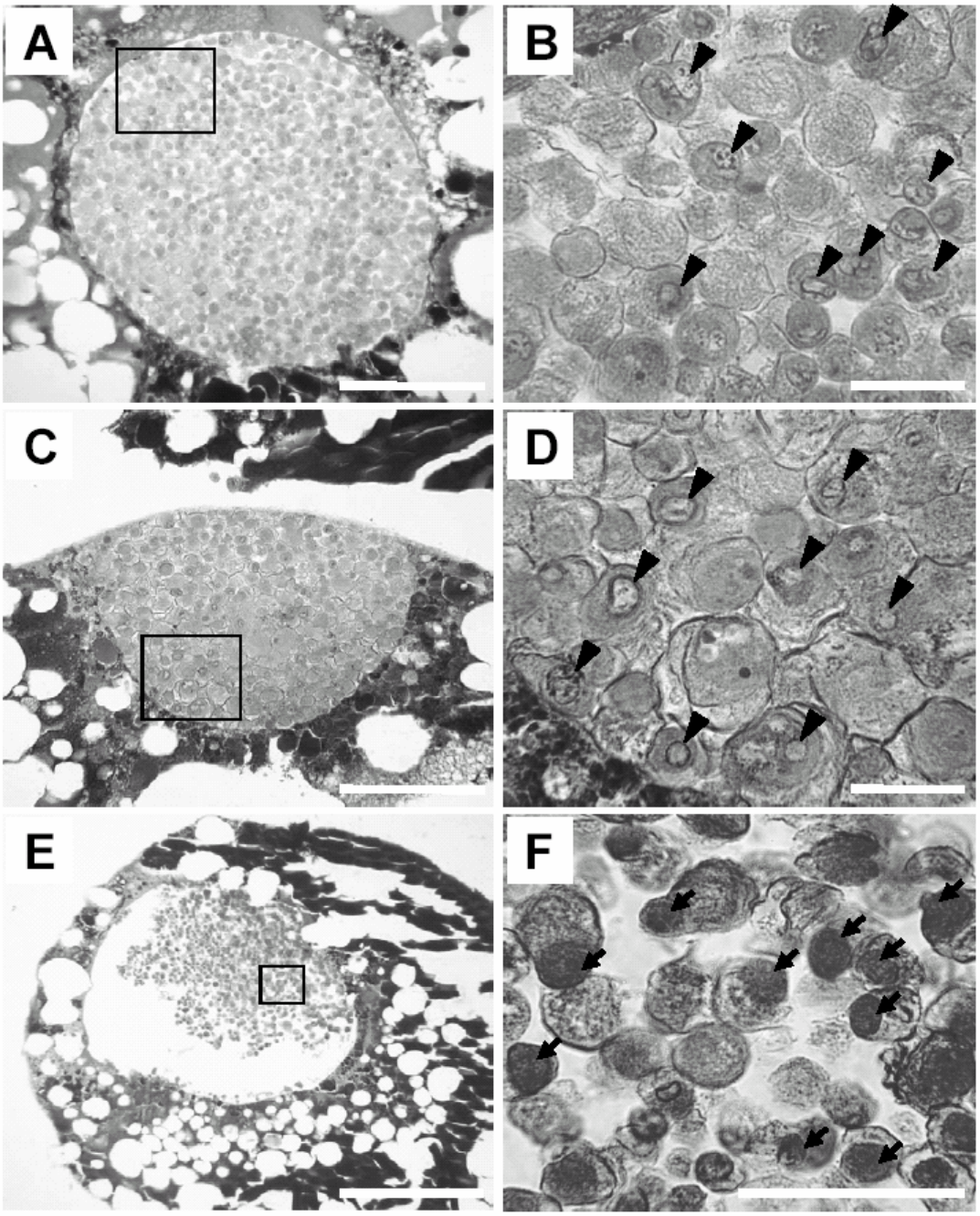

Fig. 4.

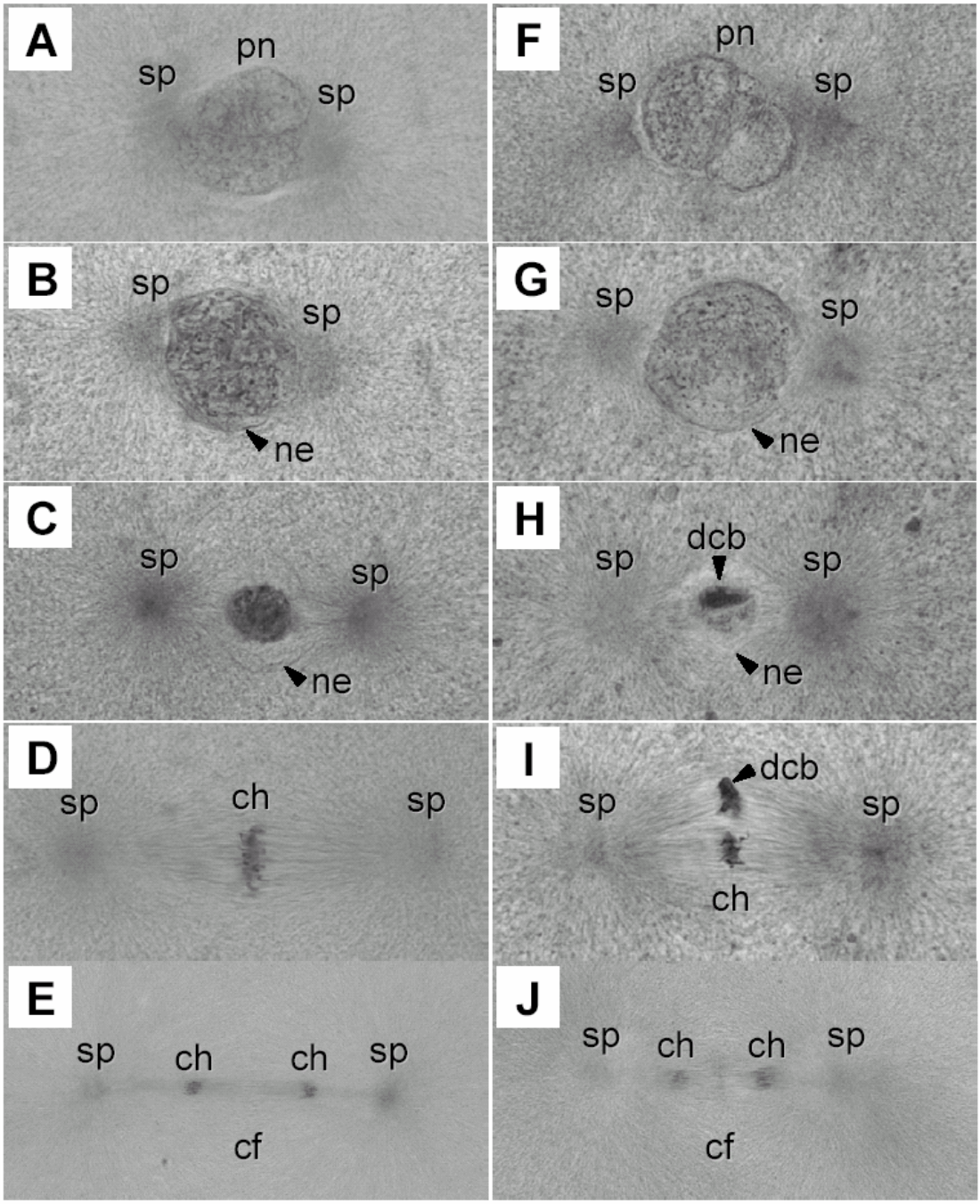
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Fig. 5.