Post-ovulatory oocyte aging induces spontaneous occurrence of polyploids and mosaics in artificial fertilization of Japanese eel, *Anguilla japonica*

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A B S T R A C T

Spontaneous polyploids and mosaics have often been observed in artificially propagated larvae of the Japanese eel, *Anguilla japonica*. However, the mechanisms responsible for such unusual cytotypes are unclear. In this study, we examined the relationship of such polyploidization and mosaicism in larvae resulting from artificial propagation to egg quality (fertilization rate and hatching rate) and viability of larvae, and then clarified the inducing factors and the mechanism for occurrence of such phenomena. Eggs stripped from females after induced maturation were artificially inseminated with sperm pre-cultured with artificial seminal plasma. Ploidy was determined by measuring the relative DNA content of the nuclei with flow cytometry. Of 968 embryos from 32 full-sib families, 9.1% were determined to be abnormal, most of which were triploids (86.5% of abnormal embryos); others were haploids (1.1%), aneuploids (2.3%), and mosaics (10.1%). The percentage of normal diploids from each family varied between 56.3% and 100% (90.9 ± 11.7%, n = 32). A significant positive correlation was found between the fertilization rate (*P* < 0.001) or the hatching rate (*P* < 0.001) and the percentage of diploids. Survival rate of triploid eels was similar to diploid eels at 10 days after fertilization whereas aneuploids were inviable. When eggs were left in the body cavity of the female for four hours after ovulation and subsequently fertilized, the percentage of diploids decreased. We tried to elucidate the cause for the occurrence of spontaneous triploids by genetic analysis using 26 microsatellite DNA markers, which have been developed and mapped in relation to the centromere. These results suggest that the occurrence of cytogenetically unusual progeny is associated with over-ripening or aging of ova caused by the lapse of time from ovulation until fertilization, and spontaneous triploid larvae are derived from the duplication of the maternal chromosome set by inhibition of the second polar body release after normal meiosis I (crossing over) in oocyte and fertilization with normal sperm.

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1. Introduction

Occurrence of spontaneous polyploids and mosaics is often identified within both farmed and wild populations of fish. These phenomena have been mainly reported in freshwater fish species, such as cyprinids (*Tinca tinca; Flajšhans et al., 1993, 2007; Noemachellus barbatulus; Collares-Pereira et al., 1995; Cyprinus carpio: Anjum and Jankun, 1994; Cherfas et al., 1991), ictalurids (*Ameiurus nebulosus: Cormier et al., 1993*), acipenserids (*Acipenser mikadoi: Zhou et al., 2011*), and salmonids (*Oncorhynchus mykiss: Aegerter and Jalabert, 2004; Cuellar and Uyeno, 1972; Thorgaard and Gall, 1979*), although little is known about these phenomena especially in marine fish as well as catadromous species like anguillids.

Japanese eel (*Anguilla japonica*) is one of the most important aquaculture species in Japan. Since stocking material for eel culture depends completely on captured wild glass eels, unstable supplies and rising glass eel prices have become serious problems for the eel culture industry. Artificially matured gametes have been successfully obtained by using hormonal treatment (*Ohta et al., 1997a*) and subsequently viable leptocephali (*Tanaka et al., 2001*) and glass eels (*Tanaka et al., 2003*) have been successfully produced by the development of appropriate rearing techniques. However, techniques for mass-producing glass eels are not yet firmly established. Unstable quality of eggs obtained from females induced to mature by hormonal treatments is one of the impediments for the mass production of glass eels.
Previously, we reported that abnormal polyploids often occur in artificially propagated eel larvae (Ohta et al., 2003). Of the abnormal polyploid larvae, most were triploid and the rest were haploid, tetraploid, pentaploid, aneuploid, and mosaic. However, the cause of polyploidization and mosaicism, and the relationship of such phenomena to fertility, hatchability, and viability of larvae are largely unknown. To determine factors underlying such cytotypes will be helpful for improving the artificial seed production techniques of the Japanese eel.

The following causes are presumably involved in the appearance of spontaneous triploid larvae; polyspermy, unreduced sperm, premeiotic endomitosis, apomixis, retention of the second polar body, and unequal cleavage. In the current protocol for obtaining eel eggs, females are weekly injected with salmon pituitary extract (SPE) to promote oocyte growth. Mature females that have full-grown oocytes need to be successively injected with SPE for priming and maturation-inducing steroid (MIS) or its precursor to induce ovulation following final oocyte maturation including resumption of meiosis and germinal vesicle breakdown (Kagawa et al., 2005). From 14 h after MIS or its precursor injection, females are checked for ovulation at 2 h intervals by applying gentle pressure on the abdomen. And then eggs are gently stripped from ovulated females and used for artificial fertilization immediately. Therefore, over-ripening of eggs caused by the lapse of time from ovulation until fertilization may be one of the causes of occurrence of abnormal ploidy levels. Additionally, sperm collected from mature males was pre-diluted with an artificial seminal plasma (Ohta and Izawa, 1996) and stored until use for artificial fertilization at 4 °C for up to a maximum period of three weeks. Thus, aging of spermatozoa caused by the lapse of time from collection until fertilization may be also one of the causes of occurrence of abnormal ploidy levels.

In the present study, we determined the ploidy level of hatching larvae obtained from 32 full-sib families using flow cytometry, and compared the fertilization and hatching rate among each family in order to elucidate the effect of abnormal ploidy larvae on such variables. We also examined the effect of aging of gametes on the fertility, hatchability and ploidy of larvae on an experimental basis. Furthermore, we examined the cause for the occurrence of spontaneous triploids by genetic analysis using 26 microsatellite DNA markers, which have been developed and mapped in relation to the centromere detailed by Nomura et al. (2006).

2. Materials and methods

2.1. Broodstock and hormonal treatment

Cultured eels purchased from a commercial farm were acclimated to seawater at the National Research Institute of Aquaculture, Mie, Japan, and then maturation was induced by hormonal treatment in flow-through tanks holding 400 l of seawater at 20 °C. Hormonal treatment was carried out for artificial maturation, as described previously (Kagawa et al., 1997; Ohta et al., 1996a). Female eels (500 to 1000 g) were repeatedly injected with SPE, followed by injection with 17α,20β-dihydroxy-4-pregnen-3-one (DHP; Sigma, St. Louis, MO, USA). Male eels (300 to 500 g) were repeatedly injected with human chorionic gonadotropin (ASKA Pharmaceutical Co. Ltd., Tokyo, Japan). The gametes were obtained by gently stripping ovulating females and mature males. Two grams of ovulated eggs was inseminated with 1 ml of pre-diluted milt (sperm motility > 80%) for determination of egg fertility and hatchability, and the remaining eggs (100 to 300 g) were inseminated with enough volume of pre-diluted milt for mass production of larvae. Eggs obtained from 32 females were used in this study. The individual females used for each experiment were identified as Nos. 1–32; see Table 1.

### Table 1

| Female ID | n  | Ploidy | Diploid | Triploid | Mosaic<sup>a</sup> | Aneuploid<sup>b</sup> |
|-----------|----|--------|---------|----------|-----------------|------------------|
| 1         | 30 | 30     | 29      | 1        | 0               | 0                 |
| 2         | 30 | 30     | 30      | 0        | 0               | 0                 |
| 3         | 30 | 30     | 24      | 5        | 0               | 1                 |
| 4         | 30 | 30     | 30      | 0        | 0               | 0                 |
| 5         | 32 | 30     | 18      | 13       | 1               | 0                 |
| 6         | 30 | 30     | 30      | 0        | 0               | 0                 |
| 7         | 30 | 30     | 30      | 0        | 0               | 0                 |
| 8         | 36 | 33     | 3        | 0        | 0               | 0                 |
| 9         | 30 | 29     | 1        | 0        | 0               | 0                 |
| 10        | 30 | 1      | 19       | 10       | 0               | 0                 |
| 11        | 30 | 22     | 8        | 0        | 0               | 0                 |
| 12        | 30 | 28     | 2        | 0        | 0               | 0                 |
| 13        | 30 | 28     | 2        | 0        | 0               | 0                 |
| 14        | 30 | 28     | 2        | 0        | 0               | 0                 |
| 15        | 30 | 30     | 0        | 0        | 0               | 0                 |
| 16        | 30 | 28     | 2        | 0        | 0               | 0                 |
| 17        | 30 | 30     | 0        | 0        | 0               | 0                 |
| 18        | 30 | 28     | 2        | 0        | 0               | 0                 |
| 19        | 30 | 21     | 5        | 4        | 0               | 0                 |
| 20        | 30 | 30     | 0        | 0        | 0               | 0                 |
| 21        | 30 | 29     | 0        | 0        | 0               | 1                 |
| 22        | 30 | 29     | 0        | 1        | 0               | 0                 |
| 23        | 30 | 26     | 4        | 0        | 0               | 0                 |
| 24        | 30 | 30     | 0        | 0        | 0               | 0                 |
| 25        | 30 | 30     | 0        | 0        | 0               | 0                 |
| 26        | 30 | 30     | 27      | 1        | 2               | 0                 |
| 27        | 30 | 30     | 28      | 2        | 0               | 0                 |
| 28        | 30 | 23     | 6        | 1        | 0               | 0                 |
| 29        | 30 | 28     | 2        | 0        | 0               | 0                 |
| 30        | 30 | 30     | 0        | 0        | 0               | 0                 |
| 31        | 30 | 30     | 0        | 0        | 0               | 0                 |
| 32        | 30 | 24     | 6        | 0        | 0               | 0                 |
| Total     | 968| 879    | 77       | 9        | 2               | Mean ± SD 909 ± 11.7 |

<sup>a</sup> 2n/3n: 1; 2n/4n: 5; 2n/3.7n: 2; 2n/3.8n: 1.
<sup>b</sup> 2.22C and 2.24C.

2.2. Determination of fertilization and hatching rates

We estimated the rates of fertilization and hatching in the eggs using a microplate method (Unuma et al., 2004) with slight modification. Just after insemination, 3 ml of seawater containing about 120 eggs was transferred to the Petri dish using a glass pipette, and the number of eggs was counted (N). The transparent eggs were stocked in 48-well microplates (Iwaki, Tokyo, Japan) with each well filled with 1 ml of filtered (pore size, 0.2 μm) seawater containing penicillin G potassium (Banyu Pharmaceutical, Tokyo, Japan) at 100,000 IU/l, streptomycin sulfate (Meiji Seika, Tokyo, Japan) at 0.1 g/l, and polyethylene glycol at 1 μg/ml. The plates were maintained at 23 °C in an incubator where humidity was kept at 100% to avoid evaporation of the rearing water. Four hours after insemination, the progress of cleavage was observed under a stereoscopic microscope. Three days after fertilization (daf), hatched larvae were counted. Fertilization and hatching rates were calculated by the following formulae:

Fertilization rate (%) = 100 × the number of eggs which were observed to show cleavage/N.

Hatching rate (%) = 100 × the number of hatched larvae/N.

2.3. Flow cytometry

Red blood cells from parental fishes and sperm from sires were fixed with 99.5% ethanol, and stored at −20 °C until analysis. Embryos and larvae obtained from the 32 females were sampled at 1, 2, 3,
10 daf and fixed in the same manner. Ploidy level was determined by flow cytometry (FCM) using the PA type flow cytometer (Partec, Germany) as described previously (Nomura et al., 2004). Briefly, 10 to 20 μl of cell suspensions prepared from fixed blood and sperm samples were re-suspended in 200 μl of extraction buffer and were incubated for 15 min at room temperature. Subsequently, 1 ml of 4',6-diamidino-2-phenylindole (DAPI) staining solution contained in the CyStain DNA 2 step high resolution DNA staining kit (Partec) was used. Larvae were individually minced in 200 μl of extraction buffer by using the tip of a pipette and incubated for 15 min at room temperature. The samples were then filtered through a 30 μm mesh filter (Partec Cell Trics disposable filter units; Partec) and 1 ml of staining solution was added. Ploidy levels of larvae were determined as follows from the relative DNA content of somatic cells when erythrocytes of parental fishes were used as a standard of normal diploidy (2C); and 0.8–1.2C for haploid; 1.8–2.2C, diploid; 2.8–3.2C, triploid; 3.8–4.2C, tetraploid; 4.8–5.2C, pentaploid; clear-double peak, mosaic; and other peak, aneuploid.

2.4. Effect of sperm preservation on egg quality and larval ploidy

Semen collected from three males, which were artificially induced to mature by hormonal treatment, was diluted 100-fold with artificial seminal plasma (K30-ASP, Ohta et al., 1997b) and stored at 4 °C for three weeks. After three weeks, we collected semen again from the same males and diluted it in the same manner. Two gram samples of eggs (3000–4000 eggs) obtained from three artificially matured females were fertilized with 1 ml of either three week stored or of fresh sperm solution (1.0–2.0 × 10⁹ spermatozoa) in combination with one female for one male. Fertilization rates and hatching rates were estimated by the microplate method described above. Thirty larvae of each group were determined for the ploidy level at 3 daf.

2.5. Effect of post-ovulatory egg aging on egg quality and larval ploidy: Experiment 1

From the 32 females induced to mature, nine females were randomly selected. Eggs were collected just after observation of ovulation, and then immediately inseminated with pre-diluted milk. We left a part of ovulated eggs in the female’s body cavity without stripping all of the eggs, and returned the females to same tank. Post-ovulatory aging eggs were again collected at 2.5 h after initial collection from four females and then at 4 h after initial collection from the remaining five females, and inseminated with the same sperm solution used for the fertilization of the freshly ovulated eggs. Fertilization rates and hatching rates were estimated by the microplate method described above. Thirty larvae of each group were determined for the ploidy level at 3 daf.

2.6. Effect of post-ovulatory egg aging on egg quality and larval ploidy: Experiment 2

Four females were randomly selected from the 32 females that were artificially induced to maturation. Females were checked for ovulation every 2 h from 14 h after DHP injection. Eggs were collected just after confirmation of ovulation. Only about half of the estimated number of eggs of each fish was stripped initially, and then immediately the females were returned to the flow-through tank holding 400 l of seawater at 20 °C. From all of the stripped eggs, 200–300 eggs were immediately fixed with 2% glutaraldehyde — 1.5% paraformaldehyde (0.1 M phosphate buffer, pH 7.4) for cytological observation, and 2 g of eggs (3000–4000 eggs) was inseminated with 1 ml of pre-diluted milk (1.0–2.0 × 10⁹ spermatozoa). Aging of ovulated eggs was performed in two ways; in vitro, and in vivo. In the in vitro experiment, the remaining eggs were divided into three reclosable polyethylene bags (85 mm × 60 mm × 0.08 mm, Seisan Nippon Sha Ltd., Japan) in aim to minimize the negative effect by oxidation and/or drying of eggs, and incubated at 22 °C until fertilization. The post-ovulatory eggs were taken out at 1, 2, and 4 h after ovulation, and then 200–300 eggs were fixed with fixing solution for observation of the nuclei of eggs, and 2 g of eggs was inseminated with the same sperm. In the in vivo experiment, the eggs, that were left in the female’s body cavity, were stripped 4 h after ovulation, and then 200–300 eggs were fixed with fixing solution for observation of the nuclei of eggs, and 2 g of eggs was inseminated with the same sperm. Fertilization rates and hatching rates were estimated by the microplate method described above. Hatched larvae were randomly collected from each group at 3 daf for ploidy analysis.

2.7. Fluorescent staining of the nuclei of the oocytes with Hoechst 33258

For examination of the nuclear state of the oocytes, the cortical cytoplasm around the animal pole was isolated from the yolk using a scalpel and forceps under a binocular microscope (×20), and then mounted on a clean hole slide glass (Toshinrich Co., Ltd., Japan). The sample was immersed in 18 M KOH, 100% xylene, 50% ethanol, and 70% ethanol each for 10 s, and then stained with 100 μg/ml Hoechst 33258 dye for 40 min at room temperature. After removal of the Hoechst dye, the sample was cleared in 2.5% propyl gallate — 97.5% Murray's clear (benzyl alcohol: benzyl benzoate = 1:2, v/v), and gently squashed with a coverslip. Thirty eggs obtained from each group were observed by fluorescence microscopy.

2.8. Genetic analysis of triploid larvae with G–c mapped microsatellite loci

Eighteen triploid larvae, which were sampled from a full-sib family at 3 daf and their ploidy level determined by FCM, were used for the genetic analysis. A DNA sample was extracted from each larva using Wizard SV Genomic DNA Purification System (Promega, USA). Genotyping was carried out using 26 microsatellite DNA markers, which have been developed and mapped in relation to the centromere by Nomura et al. (2006). Microsatellite genotypes were screened in parent fish at 26 microsatellite loci. Half-tetrad analysis can be carried out only at the locus for which the maternal genotype is heterozygous and the paternal alleles can be distinguished from the maternal alleles. The M–C recombination rate (second meiotic division segregation frequency = y) was estimated from the frequency of recombinant heterozygotes in triploid progeny at the locus that is genetically heterozygous in the female parent eel.

2.9. Statistics

Spearman’s correlation coefficient was used to elucidate the correlation between the normal diploid rate and the corresponding rates of fertilization and hatching. Kruskal–Wallis test was used to compare the percentages of each ploidy level of embryos and larvae among days after fertilization. Paired t-test was used to compare the experimental effects on egg quality and larval ploidy. Differences were accepted as significant when P < 0.05.

3. Results

3.1. Appearance of abnormally ploidy levels in the progeny obtained from artificially induced gametes

Of 968 larvae (3 daf) from 32 full-sib families, 89 larvae (9.1%) were determined to be abnormal, most of which were triploids (n = 77, 86.5% of abnormal embryos); others were haploids (n = 1, 1.1%), aneuploids (n = 2, 2.3%), and mosaics (n = 9, 10.1%) (Table 1). The percentage of normal diploids from each family varied between 56.3% and 100% (mean ± SD were 90.9 ± 11.7%). There were significant
positive correlations between the percentage of diploid larvae and the fertilization rate ($P < 0.001$) as well as the hatching rate ($P < 0.001$) (Fig. 1). As a result of FCM analysis of embryos and larvae at 1, 2, 3, and 10 daf from 11 full-sib families, the percentages of diploid, triploid, pentaploid, and mosaics were relatively stable during embryogenesis and ontogenesis, although the percentage of aneuploids decreased as a developmental stage and did not occur after 3 daf (Table 2).

3.2. Effect of sperm preservation on fertility, hatchability, and larval ploidy

As for the effect of sperm aging, although corresponding rates of fertilization and hatching were significantly higher when fertilized with semen stored for 3 weeks than when fertilized with fresh semen ($P < 0.05$), there was no significant difference in the normal diploid rate of larvae (Table 3).

3.3. Effect of post-ovulatory egg aging on fertility, hatchability, and larval ploidy

As for the effect of post-ovulatory oocytes aging, in experiment 1, corresponding rates of fertilization and hatching were reduced at 2.5 h after ovulation relative to the control, although no significant difference was observed for the normal diploid rate. At 4 h after ovulation, corresponding rates of hatching and normal diploid were significantly reduced relative to the control (Table 4; $P < 0.05$).

In experiment 2, corresponding rates of fertilization and hatching were $72.4 \pm 22.7\%$ and $54.1 \pm 25.2\%$ (mean ± SD) respectively, when eggs were fertilized just after confirmation of ovulation. When eggs were left in the body cavity of the female (in vivo) for 4 h after ovulation and subsequently fertilized, corresponding rates of fertilization and hatching were significantly reduced the same as experiment 1 (Fig. 2; $P < 0.05$). In in vitro experiment, corresponding rates of fertilization and hatching were reduced at 1 h after ovulation, and significantly reduced at after ovulation (Fig. 2; $P < 0.05$). There was no significant difference for both fertilization rate and hatching rate at 4 h after ovulation between in vivo and in vitro (Fig. 2).

As a result of examination of the nuclear state of unfertilized eggs, we observed not only normal eggs which showed normal metaphase II nuclei (Fig. 3-A), but also abnormal eggs which showed abnormal nuclei such as the spread chromosomes (Fig. 3-B), separated chromosomes (Fig. 3-C), and decondensed chromatin (Fig. 3-D). The percentage of eggs showing normal metaphase II nuclei was 92.3 ± 3.8\% just after confirmation of ovulation, whereas the percentages of normal metaphase II at 4 h after ovulation were 60.9 ± 8.4\% in in vivo, and 56.8 ± 24.8\% in in vitro. There was no significant difference at the percentages of normal metaphase II at 4 h after ovulation between in vivo and in vitro (Fig. 2).

The percentage of normal diploid larvae was 94.2 ± 5.7\% when eggs were fertilized just after confirmation of ovulation, whereas the percentage of normal diploid larvae was significantly reduced to 56.7 ± 20.2\% when eggs were left in the body cavity of the female (in vivo) for 4 h after ovulation and subsequently fertilized (Fig. 2; $P < 0.05$). Similarly, in the in vitro experiment, the percentage of normal diploid larvae was also reduced at 1 h after ovulation, and significantly reduced to 64.2 ± 13.2\% at 4 h after ovulation (Fig. 2; $P < 0.05$). There was no significant difference in the percentage of normal diploid larvae fertilized at 4 h after ovulation between in vivo and in vitro (Fig. 2). Of 600 larvae from each experimental group in experiment 2, 142 larvae (23.7\%) were determined to be abnormal, most of which were triploids ($n = 115, 81.0\%$ of abnormal larvae); others were haploids ($n = 1, 0.7\%$), aneuploids ($n = 3, 2.1\%$), and mosaics ($n = 20, 14.1\%$). There was a significant positive correlation between the percentage of non-diploid (abnormal ploidy) larvae and the percentage of eggs showing abnormal metaphase II nuclei (Fig. 4; $n = 20, r_s = 0.6528, P < 0.005$).

3.4. Genotypic segregation of triploid larvae

Of the 26 microsatellite loci screened for the half-tetrad analysis, 15 loci were heterozygous in the female parent and the male parent

![Fig. 1](image1.png)

**Fig. 1.** Relation between the normal diploid rate of hatching larvae (3 daf) and the corresponding rates of fertilization (A) and hatching (B). Thirty to thirty-six larvae obtained from 32 full-sib families were used for ploidy determination by flow cytometry analysis. Spearman’s correlation coefficient was used to elucidate the correlation between the normal diploid rate and the corresponding rates of fertilization and hatching.

| Days after fertilization | Ploidy (%)<sup>a</sup> | Triploid | Pentaploid | Mosaic | Aneuploid |
|-------------------------|------------------------|----------|------------|--------|-----------|
| 1                       | 90.6 ± 10.7            | 6.4 ± 9.5 | 0.3 ± 1.0  | 0.2 ± 1.0<sup>b</sup> | 2.4 ± 4.2<sup>c</sup> |
| 2 (hatch)               | 92.7 ± 8.0             | 6.4 ± 6.4 | 0          | 0.8 ± 2.0<sup>d</sup> | 0.3 ± 1.0<sup>e</sup> |
| 3                       | 93.3 ± 8.3             | 5.8 ± 7.6 | 0          | 0.9 ± 2.2<sup>f</sup> | 0         |
| 10 (yolk absorption)    | 94.2 ± 9.1             | 5.2 ± 8.2 | 0.3 ± 1.0  | 0.3 ± 1.0<sup>g</sup> | 0         |
| Cruskal–Wallis test     | n.s                    | n.s      | n.s        | n.s    | n.s       |

Mean ± S.D., $n = 11$.

<sup>a</sup>Based on relative DNA content measured by flow cytometry. Observed cell types of seven mosaic individuals were $4n/2n$: 1; $2n/3n$: 1; $2n/4n$: 1; $2n/3n$: 1; $2n/3n$: 1; $2n/4n$: 1; $2n/4n$: 1, and observed relative DNA content of nine aneuploids were $1.46C, 1.44C, 1.64C, 1.58C, 2.32C, 2.78C, 2.62C, 2.34C, and 2.3C.

| Sperm | Fertilization (%) | Hatching (%) | Diploid (%) |
|-------|-------------------|--------------|-------------|
| Fresh | 52.9 ± 8.3<sup>a</sup> | 34.7 ± 4.1<sup>b</sup> | 94.4 ± 5.1<sup>c</sup> |
| 3 week storage | 74.7 ± 9.0<sup>a</sup> | 51.9 ± 3.5<sup>b</sup> | 94.4 ± 5.1<sup>c</sup> |

Mean ± S.D., $n = 3$.

Different superscripts in the column indicate significant difference (t-test, $P < 0.05$).
had alleles that could be distinguished from the maternal alleles. At these 15 loci, although duplication of the maternal alleles was often observed in triploid offspring, no duplication of the paternal alleles was observed (Table 5). This result indicates that these 18 triploid larvae consist of two sets of the maternal genome and a set of the paternal genome. Furthermore, the microsatellite–centromere recombination frequencies (second division segregation frequencies = y) for the 15 loci, which can be estimated from the frequency of recombinant heterozygous genotype in the triploid progeny of the heterozygous female parent, were in good agreement with the expected values, which were estimated by using four triploid families produced by inhibition of the second polar body release (Table 5). These results strongly suggest that at least the 18 triploid larvae examined in this study were derived from duplication of the maternal genome by inhibition of the second polar body release after normal meiosis I (crossing over) in the oocyte and then fertilized with normal sperm.

4. Discussion

In the present study, we determined the ploidy level of hatching larvae obtained from 32 full-sib families using flow cytometry. As a result, approximately 90% of larvae were normal diploid, and most of the remaining 10% of larvae were triploids. These results are in good agreement with the results of our previous study (Ohta et al., 2003). Therefore, these phenomena are reproducible in artificially propagated larvae of the Japanese eel as long as the maturation, induction and the artificial fertilization are conducted according to the current protocol.

As a result of FCM analysis, triploids, pentaploid, and mosiacs were relatively stable during early developmental stages, although the percentages of aneuploids decreased with the proceeding of development and did not occur after 3 daf (Table 2).

This result indicated that both polyploid and mosaic larvae are viable, whereas aneuploids cannot survive. It is well known that polyploids, especially triploids, have a normal survival capacity in many fish species (Piferrer et al., 2009). Spontaneous triploids observed in the artificial propagated eel larvae also seem to be as viable as diploids. Generally, spontaneous triploids are expected to be sterile due to interference with gametogenesis, resulting in vestigial or highly delayed gonadal development, or to be at least infertile due to random segregation of trivalents producing aneuploid gametes incapable of fertilization (Piferrer et al., 2009).

In the experiment that investigated the effect of sperm aging on fertility, hatchability, and ploidy levels of hatching larvae, corresponding rates of fertilization and hatching were significantly higher when fertilized with semen stored for 3 weeks than when fertilized with fresh semen (P < 0.05; Table 3), whereas there was no significant

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Table 4

|                      | Short storage (n = 4) | Long storage (n = 5) |
|----------------------|----------------------|----------------------|
|                      | Freshly ovulated 2 h | Freshly ovulated 4 h |
| Fertilization (%)    | 87.0 ± 16.6a         | 85.3 ± 12.2a         |
| Hatch (%)            | 64.3 ± 30.5a         | 71.4 ± 13.4a         |
| 2n % (3 daf)         | 95.8 ± 5.0a          | 97.3 ± 3.7a          |

Mean ± S.D.

Different superscripts in the line indicate significant difference (t-test, \( P > 0.05 \)).

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Fig. 2. Effect of post-ovulatory oocyte aging on the corresponding rates of fertilization (A), hatching (B), larvae (3 daf) with normal diploid (C), and unfertilized eggs showing normal metaphase of the second meiotic division (D) in experiment 2. Values represent the mean ± standard errors for eggs from 4 females. Aging of ovulated eggs was performed in two ways; in vitro, and in vivo. In the in vitro experiment, the eggs were divided into three polyethylene bags and incubated at 22 °C until fertilization. In the in vivo experiment, the eggs were left in the female’s body cavity and stripped 4 h after ovulation. Thirty eggs obtained from each experimental group (n = 20; five groups × four females) were observed by fluorescence microscopy (Fig. 3), and thirty larvae (3 daf) obtained from each experimental group (n = 20) were used for ploidy determination by flow cytometry analysis.
difference for the normal diploid rate in the hatching larvae. The motility of spermatozoa in the milt has been often very low just after the collection of milt, but the percent motility of these spermatozoa increased by incubation in the artificial seminal plasma (ASP) (Ohta et al., 1997b). Therefore, fertilization and hatching rates in this experiment were presumably caused by the difference of the sperm motility with or without incubation in the ASP after the collection of milt. Thus, we conclude that the storage of spermatozoa is not a cause of the polyploidization in eel larvae.

In general, post-ovulatory oocyte aging has been suggested as one of the main causes of poor egg quality in many fish species (Kjørsvik et al., 1990). In the Japanese eel, Ohta et al. (1996b) reported that fertility of eggs retained in the body cavity decreased rapidly after ovulation. Our two experiments show similar results to the previous study. However, little is known about cytological changes caused by the aging of post-ovulatory oocytes in fish. In rainbow trout *Oncorhynchus mykiss*, Aegerter and Jalabert (2004) reported that the survival rate decreased and malformation rate and spontaneous triploid and mosaic rate increased with an increase in post-ovulatory oocyte aging. Our data also suggest that the spontaneous triploid and mosaic rate increased with an increase in post-ovulatory oocyte aging when the ovulated eggs had been left either in vivo or in vitro prior to fertilization. Furthermore, we observed that the percentage of eggs that showed normal metaphase II nuclei decreased with an increase in post-ovulatory oocyte aging by fluorescence microscopy. We observed that abnormal eggs showed abnormal nuclei such as spread chromosomes, separated chromosomes, and decondensed chromatin. Although the mechanism of such an abnormality is unknown, it is suggested that the increase of such cytologically abnormal eggs was closely linked to the decrease of fertility as well as increase of triploid and mosaics.

In this study, we suggest that spontaneous triploid larvae occur by the duplication of the maternal genome due to the inhibition of the second polar body release after normal meiosis I (crossing over) in oocyte and subsequent fertilization with normal sperm, based on the genetic analysis using microsatellite markers located in the distal region of chromosomes.

In conclusion, occurrence of cytogenetically unusual progeny occurs in association with over-ripening or aging of eggs caused by the
Table 5

| Locus   | G-C distance (cm) | Parental genotype (bp) Total | Genotypes of spontaneous triploid larvae | Total | Recombination frequency (y) |
|---------|------------------|-----------------------------|----------------------------------------|-------|---------------------------|
| Aj/D     | 0.4              | 142/140 136/134             | a/b/c 0 0 2 10 3 3                | 18    | 0.000 0.008 0.144         |
| Aj/t     | 3.9              | 110/100 115/113             | a/b/d 0 0 5 4 5                  | 18    | 0.000 0.078 1.404         |
| Aj/g     | 8.0              | 115/99 117/137              | a/c/d 1 – 7 – 10 –                | 18    | 0.056 0.160 1.227         |
| Aj/Tr    | 14.1             | 135/129 138/128             | b/c/d 0 5 2 7 3 1                | 18    | 0.278 0.282 0.001         |
| Ang/14   | 33.3             | 116/98 133/107              | 10 7 1 0 0 0                     | 18    | 0.944 0.666 2.095         |
| Aj/Tr    | 35.6             | 140/114 145/133             | 6 7 1 0 0 3 1                   | 18    | 0.722 0.712 0.003         |
| Aj/Tr    | 37.5             | 208/138 174/126             | 6 9 3 0 0 0 0                   | 18    | 0.933 0.750 0.167         |
| Aj/Tr    | 39.1             | 130/114 115/131             | 8 7 1 0 1 1 1                  | 18    | 0.833 0.782 0.061         |
| Aj/Tr    | 40.0             | 112/102 120/108             | 6 12 0 0 0 0 0                  | 18    | 1.000 0.800 0.900         |
| Aj/Tr    | 42.6             | 111/109 131/117             | 8 8 1 0 0 0 0                   | 18    | 0.944 0.852 0.181         |
| Aj/Tr    | 42.9             | 127/117 107/107             | 12 7 1 0 1 4 4                 | 18    | 0.722 0.858 0.387         |
| Aj/Tr    | 45.2             | 161/155 137/137             | 15 12 3 0 0 0 0                 | 18    | 0.833 0.904 0.099         |
| Aj/Tr    | 45.5             | 19/101 124/110              | 10 7 1 0 0 0 0                  | 18    | 0.944 0.921 0.014         |
| Aj/Tr    | 46.9             | 78/85 93/83                 | 8 9 0 0 0 0 1                  | 18    | 0.944 0.938 0.001         |
| Aj/Tr    | 48.4             | 142/138 145/135             | 9 8 0 1 0 0 0                 | 18    | 0.944 0.968 0.010         |

References

Aegerter, S., Jalabert, B., 2004. Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout, Oncorhyncus mykiss. Aquaculture 231, 59–71.

Anjum, R., Jankun, M., 1994. Spontaneous triploid common carp (Cyprinus carpio L.) in a farm population. Cybotes 78, 153–157.

Cherfas, N.B., Rothbard, S., Hulata, G., Kozinsky, O., 1991. Spontaneous diploidization of male cyprinids. Marine Biology 109, 207–213.

Cormier, S.M., Neiheisel, T.W., Williams, D.E., Tiersch, T.R., 1993. Natural occurrence of spontaneous triploidy in a wild brown bullhead. Transactions of the American Fisheries Society 122, 390–392.

Collares-Pereira, M.I., Madeira, J.M., Rab, P., 1995. Spontaneous triploidy in the stone loach, Misgurnus anguillicaudatus (Bloch). Aquaculture 137, 115–122.

Cormier, S.M., Neiheisel, T.W., Williams, D.Q., Tiersch, T.R., 1993. Natural occurrence of triploidy in a wild brown bullhead. Transactions of the American Fisheries Society 122, 390–392.

Cuelar, O., Uyeno, T., 1972. Triploidy in rainbow trout. Cytopgenetics 11, 508–515.

Nakajima, J., Kagawa, H., Tanaka, H., Nomura, K., Hirose, K., 2006. Mitotic induction of triploidy in Japanese eel. Aquaculture 257, 53–67.

Okamoto, N., Iwamoto, Y., Kato, H., 1996. Dilution for cool storage of the Japanese eel (Anguilla japonica) spermatozoa. Aquaculture 142, 107–118.

Ohta, H., Kagawa, H., Tanaka, H., Kato, H., 1996a. Induction of triploidy in the Japanese eel (Anguilla japonica) by half-tetrad analysis using induced triploid families. Aquaculture 257, 53–67.