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

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ABSTRACT

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 mosaicisms 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.

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; *Noemacheilus 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

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eels (Kagawa et al., 2001). Hatchability and viability of larvae are highly variable among batches of eggs from different females (Unuma et al., 2004, 2011).

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 mosaicisms, 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
Ploidies observed by flow cytometry analysis of eel larvae (3 daf) obtained from 32 full-sib families.

Female ID	*n*	Ploidy					Diploid (%)
		Haploid	Diploid	Triploid	Mosaic[a]	Aneuploid[b]	
1	30	0	29	1	0	0	96.7
2	30	0	30	0	0	0	100.0
3	30	0	24	5	0	1	80.0
4	30	0	30	0	0	0	100.0
5	32	0	18	13	1	0	56.3
6	30	0	30	0	0	0	100.0
7	30	0	30	0	0	0	100.0
8	36	0	33	3	0	0	91.7
9	30	0	29	1	0	0	96.7
10	30	1	19	10	0	0	63.3
11	30	0	22	8	0	0	73.3
12	30	0	28	2	0	0	93.3
13	30	0	28	2	0	0	93.3
14	30	0	28	2	0	0	93.3
15	30	0	30	0	0	0	100.0
16	30	0	28	2	0	0	93.3
17	30	0	30	0	0	0	100.0
18	30	0	28	2	0	0	93.3
19	30	0	21	5	4	0	70.0
20	30	0	30	0	0	0	100.0
21	30	0	29	0	0	1	96.7
22	30	0	29	0	1	0	96.7
23	30	0	26	4	0	0	86.7
24	30	0	30	0	0	0	100.0
25	30	0	30	0	0	0	100.0
26	30	0	27	1	2	0	90.0
27	30	0	28	2	0	0	93.3
28	30	0	23	6	1	0	76.7
29	30	0	28	2	0	0	93.3
30	30	0	30	0	0	0	100.0
31	30	0	30	0	0	0	100.0
32	30	0	24	6	0	0	80.0
Total	968	1	879	77	9	2	Mean ± SD 90.9 ± 11.7

[a] 2n/3n: 1; 2n/4n: 5; 2n/3.7n: 2; 2n/3.8n: 1.
[b] 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:

$$\text{Fertilization rate}\,(\%) = 100 \times \text{the number of eggs which were observed to show cleavage}/N.$$

$$\text{Hatching rate}\,(\%) = 100 \times \text{the number of hatched larvae}/N.$$

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,