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Viable Hyperdiploid Progeny between Diploid Female and Induced Triploid Male in the Loach, *Misgurnus anguillicaudatus*

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Abstract: Eggs of normal diploid (2n) loach were fertilized with spermatozoa of induced triploid (3n) loach. The resultant 2n×3n progeny showed lower survival and higher incidence of abnormality in larval stage than the control 2n × 2n cross, but a small number of individuals survived. Chromosome observation showed hyperdiploidy of the 2n×3n fry. DNA content of the two-month-old survivors measured by flow cytometry showed that they were viable 2.2 to 2.5n aneuploids.

Key words: Aneuploid; Triploid; Hyperdiploid; *Misgurnus*

In commercially important aquatic animals, mating experiments between normal diploids and artificially induced triploids have been conducted with plaice[1], rainbow trout[2-4], grass carp[5], Pacific oyster[6], and pearl oyster[7]. These studies indicated low viability of the resultant progeny in which aneuploidy was confirmed[4-7] or estimated[1-3].

In induced triploids of the loach, *Misgurnus anguillicaudatus*, females were sterile, whereas males generated aneuploid spermatozoa with a mode of 1.3n[8]. The progeny of induced triploid males showed aneuploidy with a broad distribution of chromosome number or DNA content between triploidy (3n = 75) and tetraploidy (4n = 100) when crossed to tetraploid female[8]. These hypertriploid to hypotetraploid loach exhibited poor survival potential, but a small number of fry which began to feed were able to survive[8]. However, survival capacity has not been examined in the aneuploid loach with hyperdiploid to hypotriploid chromosome number.

In the present study, we fertilized eggs of a normal diploid with spermatozoa of the induced triploid male so as to produce aneuploid progeny with chromosome number between diploidy and triploidy. Then, we observed survival rates and determined the ploidy status by counting chromosomes at the larval stage and measuring DNA content of somatic cells by flow cytometry at the two-month-old juvenile stage.

Materials and Methods

The triploids were induced by inhibiting the second polar body extrusion with hydrostatic pressure shock in 1991 (family #1058) as described in Zhang and Arai[9]. Diploid female was used from the specimens collected from the waterway in Memanbetsu Town, Hokkaido in July, 1998. Diploid male was used from the specimens collected in Kasai waterway, Shimokawasaki, Hanyu City, Saitama Prefecture in July, 1998. The ploidy of each parental fish was confirmed by measuring DNA content of erythrocytes by flow cytometry according to Zhang and Arai[9].

In July, 1998, an induced triploid male was sacrificed to take testes 10h after injection of HCG (human chroinic gonadotropin, Teikoku Zouki Co. Ltd.) accoring to Zhang and Arai[8]. A part of its testes was subjected to flow

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Table 1. Survival and abnormal rates of the progeny of induced triploid male loach

Cross	No. of eggs	Survival (%)				Abnormal (%) in 7-day-old fry
		24h*	48h	72h	7d	
2n×2n	592	85	80.4	75	52	4.4
2n×3n	1083	46	29.5	20	14	76.3

* Time (h, hours; d, days) elapsed after fertilization.

cytometry to determine ploidy status of spermatozoa. The other part was used to fertilize eggs from a normal diploid female (2n × 3n cross). Ovulation was stimulated by the injection of HCG according to Suzuki and Yamaguchi[10]. The eggs of the same female were also fertilized with spermatozoa of normal diploid male as control (2n×2n cross).

The survival rates of the 2n×3n and 2n×2n control progeny were calculated relative to number of eggs used, at 24h, 48h (hatching), 72h and 7 days after fertilization (the beginning of feeding). Number of normal feeding fry was recorded at 7 days after fertilization by observing the external appearence.

Chromosome preparation was conducted in 48-h-old embryo which yolk sac was mechanically removed according to the method described by Inokuchi *et al.*[11] with some modifications in the concentration of colchicinization (0.01%) and in the use of 0.075M potassium chloride for hypotonic treatment instead of 0.8% trisodium citrate dehydrate. Chromosome number was counted on printed photographs of metaphase spreads with high quality. Karyotype analysis was made according to Levan *et al.*[12].

For measurement of DNA content, gills of juveniles and testis of parental fish were minced finely with forceps in two drops of Eagle's MEM medium (Nissui Co. Ltd.) in 1.5 m*l* microfuge tube, and then prepared to measure DNA content by flow cytometry according to Zhang and Arai[9]. The preparation of erythrocytes was also followed Zhang and Arai[9]. Aneuploid status was approximately estimated by calculating the ratio between modal channel number of putative aneuploid individual and that of a control diploid individual, as reported in the previous work[8]. The DNA contents of samples were analyzed by excitation at 488 nm laser with Becton Dickinson FACS Calibur flow cytometer.

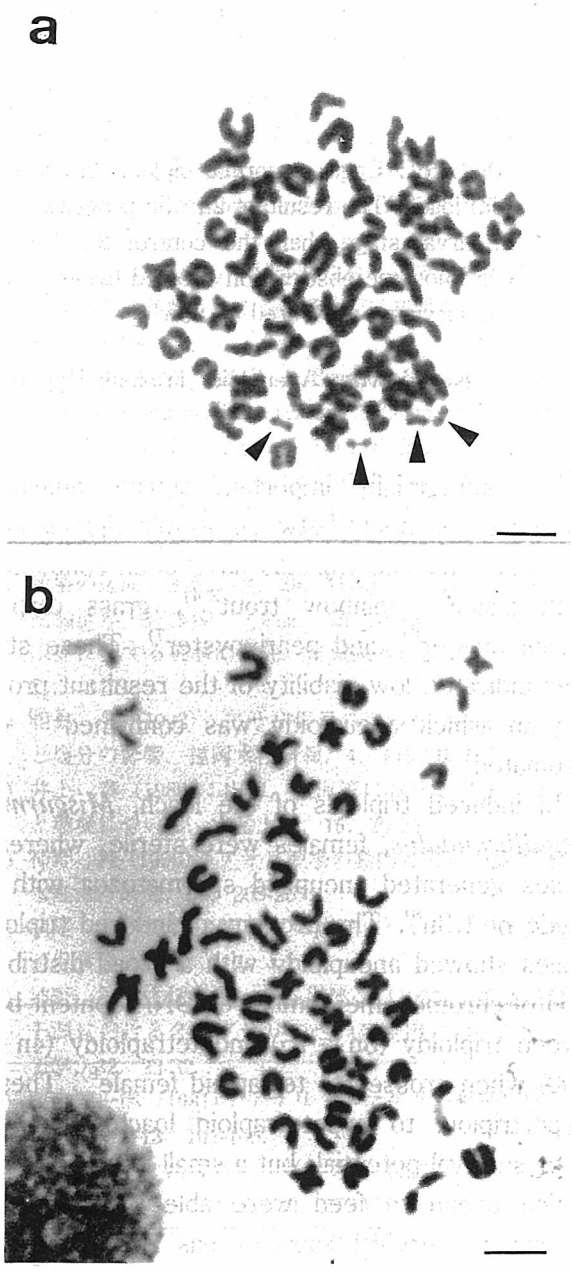

Fig. 1. Chromosome spreads of aneuploid loach embryos between normal diploid and induced triploid. a, aneuploid cell with 56 chromosomes and 4 B-chromosomes observed in #4 embryo; b, aneuploid cell with 57 chromosomes observed in #8 embryo. Scale indicates 5 μm. Arrowhead indicates B-chromosome.

Results

Table 1 shows survival rates of the 2n × 2n and 2n×3n progeny at 24h, 48h, 72h and 7 days after fertilization. The 2n × 3n cross revealed lower survival rates than the control at all the stages examined and 76% of the resultant feeding fry were abnormal. These results indicated low viability of the 2n×3n progeny in early developmental stages.

At two months after fertilization five loaches (1.6% relative to total hatched fry) in the 2n×3n cross were still alive, while eight (1.7% relative to hatched fry) in the control 2n×2n cross. Unusually low survival rate of the control resulted from a heavy mortality due to deterioration of water quality of the tank after the beginning of feeding. The progeny from 2n × 3n cross (total length; 19.7 to 36.3 mm, average 22.5 mm) exhibited abnormalities in external appearance such as shortened body, ill-developed tail, and/or bending of head and body to the left and right. No abnormal individuals were detected in surviving eight control diploids (total length; 28.2 to 35.6 mm, average 31.9 mm).

Chromosome preparations from the 2n × 3n cross revealed that all the fry were aneuploids (Fig. 1), while the control was eudiploid (2n = 50) as reported in the earlier works[13-16]. Table 2 shows the distribution of chromosome number in two-day-old fry from the 2n × 3n cross. Although chromosome number ranged from 49 to 62, the distribution of an individual mode could be categorized into three groups: around 50 to 51 (2.0 to less than 2.1n; #1,2), 54-57 (2.2n to 2.3n; #3-8), and 59 (2.4n; #8). The 2n × 3n fry #8 might be a mosaic individual with two or three different modes. No fry with hypotriploid chromosome numbers (65; 2.6n to 74; 2.9n) were observed.

Karyotype analysis indicated that each 2n×3n individual had two sets of homologous chromosomes and different number of supernumerary chromosomes (Fig. 2). Members of supernumerary chromosomes were different from individual to individual. Four of eight fry from 2n × 3n cross exhibited somatic cells with one to six micro B-chromosomes (Fig. 2, Table 2). All these micro B-chromosomes were apparently different in size and shape from normal member of the diploid loach karyotype.

Flow-cytometric analysis of testicular cells collected from the induced triploid male revealed the presence of aneuploid spermatozoa with 1.3C DNA content as reported in the previous work[8], whereas normal diploid showed haploid spermatozoa with just 1.0C DNA content (Figure not shown).

DNA content of somatic cells was also examined in two-month-old survivors from the 2n × 3n cross by flow cytometry (Fig. 3). Of five survivors examined, three individuals showed relative DNA content around 2.2C, the fourth individual had about 2.3C and the fifth had about 2.5C. Thus, all two-month-old juveniles were concluded to be viable 2.2n to 2.5n hyperdiploids. Two juveniles from the 2n × 2n control cross had just 2.0C DNA content, indicating diploidy.

Table 2. Chromosome numbers in the 48-h-old embryos of the 2n×3n cross

Embryo	Chromosome number															B-chromosome number						
#	49	50	51	52	53	54	55	56	57	58	59	60	61	62	Total	1	2	3	4	5	6	Total
1	1	2	1												4							0
2			2												2							0
3						2	1	1							4							0
4						2	3	2							7	1	1		1			3
5							1	2	1						4							0
6								1	1						2	1						1
7				1	1			1	3						6		1	1				2
8					1	2	3		2		2	1	1	1	13					1	1	2
Total	1	2	3	1	2	6	8	7	7	0	2	1	1	1	42	2	2	1	1	1	1	8

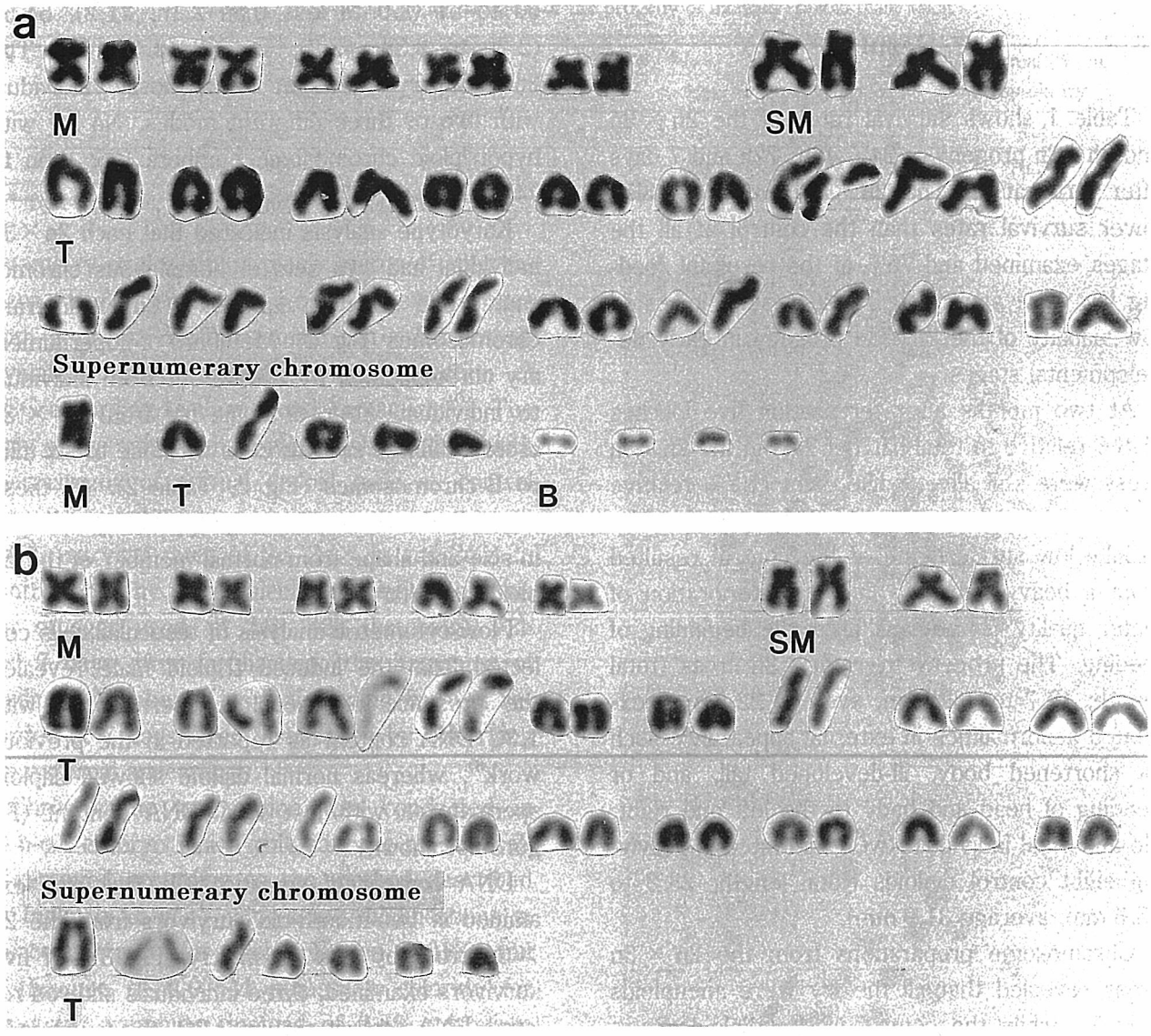

Fig. 2. Karyotypes of aneuploid loach embryos between normal diploid and induced triploid. a, karyotype based on Fig. 1a including normal diploid chromosomes (2n=50), supernumerary 6 chromosomes, and 4 B-chromosomes; b, karyotype based on Fig. 1b including normal diploid chromosomes (2n = 50) and supernumerary 7 chromosomes. M, metacentric chromosome; SM, submetacentric chromosome; T, telocentric chromosome; B, B-chromosome.

Discussion

We showed that all the 2n × 3n progeny examined were 2.1n to 2.5n aneuploids based on the chromosome counting and the flow-cytometrical DNA measurement. These progeny must be produced by the fertilization of normal haploid (1n) eggs with hyperhaploid (1.1n to 1.5n) spermatozoa. Five out of eight fry (63%) and four out of five juveniles (80%) were 2.2 to 2.3 n aneuploids. These results can be well explained by the fertilization with spermatozoa of induced triploids, which showed modal DNA content at 1.3C as reported in the present and previous work[8]. The modal 1.3C spermatozoa and the occurrence of hyperdiploid progeny in the 2n × 3n cross suggested selective production of more hyperhaploid (1.1 to 1.5n) spermatozoa than hypodiploid (1.6 to 1.9n) ones. More incidence of hypertriploids than hypotetraploids were also observed in the previous 4n × 3n cross[8]. Such a shift toward the lower aneuploidies has been elucidated by elimination of some unsynapsed univalents or by failure of hypodiploid spermatids with higher numbers

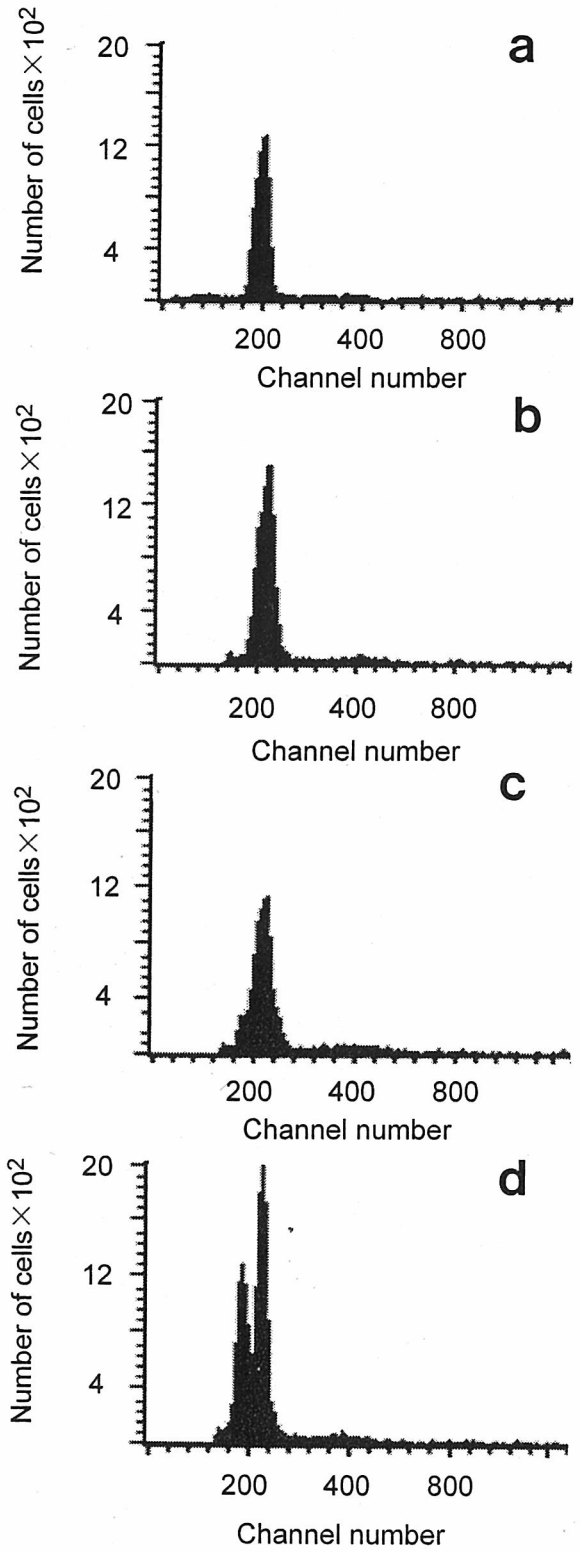

Fig. 3. Flow cytometric histograms of somatic cell suspension of control (a) and aneuploid (b-d) loaches. a, normal diploid cells with 2C DNA content; b, aneuploid cell with approximately 2.2C DNA content; c, aneuploid cell with approximately 2.3C DNA content; d, Mixture of aneuploid cells with approximately 2.5C DNA content and normal diploid cells as standard with 2C DNA content.

of extra chromosomes to differentiate into spermatozoa[17,18].

In the 2n × 3n loach, we detected supernumerary micro-chromosomes in several progeny. They are likely to be B-chromosomes because of their extremely smaller sizes than normal member of the diploid loach karyotype as well as inter- and intra-individual variation in their number[19]. The occurrence of such B-chromosome like micro-chromosomes were also reported in the 4n × 2n progeny[8]. Thus, these micro-chromosomes are considered to be paternally derived from the triploid male induced by hydrostatic pressure treatment on normally fertilized eggs. Chromosome fragmentation due to hydrostatic pressure treatment was reported by Yamazaki and Goodier[20]. However, spontaneous origin of B-chromosomes cannot be eliminated because the loaches with supernumerary micro-chromosomes were found*3.

In the 2n×3n cross, 86% of the progeny died before the beginning of feeding and 76% of surviving fry were abnormal. However, five survivors were verified to be hyperdiploids (2.2n to 2.5n) when they were sacrificed to examine ploidy status at two months after fertilization. These observations revealed that the majority of 2n × 3n loach were inviable due to probable selection against certain chromosome numbers and/or karyotypes, but some hyperdiploids were able to survive longer. The occurrence of aneuploid survivers contrasts to the complete inviability of 2.5n progeny reported in the 2n×3n cross of rainbow trout[4] and other animals[5-7].

Zhang and Arai[8] reported that relatively large number of progeny from 4n×3n cross were viable in the loach. When we compare the rate of normal fry, the previous 4n × 3n crosses gave better rates (77 to 90% relative to 100% 4n×4n control) than the present 2n × 3n (25% relative to 100% 2n × 2n control). This suggests better survival potential of hypertriploids from 4n × 3n than hyperdiploids from 2n × 3n. Similar phenomenon was observed in the progeny between diploid rainbow trout females and allotriploid

*3 Zhang, Q. and K. Arai: Abstract of 6th International Symposium on Genetics in Aquaculture, July, 1997, Stirling, Scotland.

(rainbow trout × brook trout) males; 2.5n was inviable and 3.5n restored viability[21].

As shown in the present and previous study[8], some aneuploid progeny of induced triploid males are viable in the loach. Thus, triploidy seems to be a good source of aneuploid gametes to produce viable aneuploids which will open new possibility in fish genetics. Aneuploidy, such as trisomics or other hyperdiploids with a few additional chromosomes, allows the rapid location of the gene loci to specific chromosomes as well as the phenotypic effect of individual chromosomes.

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References

1) Lincoln, R. F. (1981): Sexual maturation in triploid male plaice (*Pleuronectes platessa*) and plaice × flounder (*Platichthys flesus*) hybrids. *J. Fish Biol.*, **19**, 415-426.
2) Lincoln, R. F. and A. P. Scott (1984): Sexual maturation in triploid rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.*, **25**, 385-392.
3) Okada, H. (1987): Artificial sex control and sterility in rainbow trout. *Suisanikushu* (*Fish Genet. Breed. Sci.*), **12**, 1-16 (in Japanese).
4) Ueda, T., M. Sawada, and J. Kobayashi (1987): Cytogenetical characteristics of the embryos between diploid female and triploid male in rainbow trout. *Jpn. J. Genet.*, **62**(5), 461-465.
5) Van Eenennaam, J. P., R. K. Stocker, R. G. Thiery, N. T. Hagstrom, and S. I. Doroshov (1990): Egg fertility, early development and survival from crosses of diploid female × triploid male grass carp (*Ctenopharyngodon idella*). *Aquaculture*, **86**, 111-125.
6) Akashige, S. (1990): Growth and reproduction of triploid Japanese oyster in Hiroshima Bay, in "Advances in Invertebrate Reproduction 5" (ed. by M. Hoshi and O. Yamashita), Elsevier, Amsterdam, pp.461-468.
7) Komaru, A. and K. T. Wada (1994): Meiotic maturation and progeny of oocytes from triploid Japanese pearl oyster (*Pinctada fucata martensii*) fertilized with spermatozoa from diploids. *Aquaculture*, **120**, 61-70.
8) Zhang, Q. and K. Arai (1999): Aberrant meioses and viable aneuploid progeny of induced triploid loach (*Misgurnus anguillicaudatus*) when crossed to natural tetraploids. *Aquaculture*, **175**, 63-76.
9) Zhang, Q. and K. Arai (1996): Flow cytometry for DNA contents of somatic cells and spermatozoa in the progeny of natural tetraploid loach. *Fisheries Sci.*, **62**(6), 870-877.
10) Suzuki, R. and M. Yamaguchi (1975): Influence of water temperature on inducing spawning by hormone injection in the loach, cyprinid fish. *Suisanzoshoku*, **22**(3,4), 135-139 (in Japanese).
11) Inokuchi, T., S. Abe, E. Yamaha, F. Yamazaki, and M. C. Yoshida (1994): BrdU replication banding studies on the chromosomes in early embryos of salmonid fishes. *Hereditas*, **121**, 255-265.
12) Levan, A., K. Fredga, and A. A. Sandberg (1964): Nomenclature for centromeric position on chromosomes. *Hereditas*, **52**, 201-220.
13) Ojima, Y. and S. Hitotsumachi (1969): Cytogenetical studies in loaches (Pisces, Cobitidae). *Zool. Mag.*, **78**(4), 139-141.
14) Ojima, Y. and A. Takai (1979): The occurrence of spontaneous polyploid in the Japanese common loach, *Misgurnus anguillicaudatus*. *Proc. Japan Acad.*, **55**. Series B, 487-491.
15) Arai, K., K. Matsubara, and R. Suzuki (1991): Karyotype and erythrocyte size of spentaneous tetraploidy and triploidy in the loach *Misgurnus anguillicaudatus*. *Nippon Suisan Gakkaishi*, **57**(12), 2167-2172.
16) Kusunoki, T., K. Arai, and R. Suzuki (1994): Viability and karyotypes of interracial and intergeneric hybrids in loach species. *Fisheries Sci.*, **60**(4), 415-422.
17) Fankhauser, G. and R. R. Humphrey (1950): Chromosome number and development of progeny of triploid axolotl females mated with diploid males. *J. Exp. Zool.*, **115**, 207-250.
18) Fankhauser, G. and R. R. Humphrey (1954): Chromosome number and development of progeny of triploid axolotl males crossed with diploid females. *J. Exp. Zool.*, **126**, 33-58.
19) Jones, R. N. and H. Rees (1982): B-chromosomes. Academic Press, New York, 266p.
20) Yamazaki, F. and J. Goodier (1993): Cytogenetic effects of hydrostatic pressure treatment to suppress the first cleavage of salmon embryos. *Aquaculture*, **110**, 51-59.
21) Ueda, T., R. Sato, M. Iwata, A. Komaru, and J. Kobayashi (1991): The viable 3.5n trouts produced between diploid females and allotriploid males. *Jpn. J. Genet.*, **66**(1), 71-75.

ドジョウの二倍体雌と三倍体雄間の生存性高二倍体子孫

荒井克俊・稲森由絵

正常二倍体の卵を人為三倍体の精子で受精したところ，その結果生じる二倍体×三倍体の子孫は低い生存率と高い奇形率を仔稚魚期に示した。しかし，少数個体は摂餌を開始し，さらに生存した。受精2日令の胚の染色体観察から，これらは高二倍性異数体であることが判った。2月令の生残個体のDNA量フローサイトメトリーの結果はこれらが生存性の高二倍体（2.2n～2.5n）であることを示した。