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Effects of Ultraviolet Irradiation on Genetical Inactivation and Morphological Structure of Sperm of the Pacific Abalone *Haliotis discus hannai*

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Summary

Effects of ultraviolet (UV) irradiation on genetical inactivation and morphological structure of sperms were examined in the Pacific abalone. Irradiation for 20 s at a UV intensity of 720 μW/cm^2/s was the optimum dose to achieve haploid gynogenesis. The fertilization rate apparently decreased with increasing irradiation time, and the development of the eggs fertilized with the genetically inactivated sperms terminated before reaching the veliger stage. Scanning electron microscopy showed clear destruction of the sperm acrosome and flagellum in the UV-irradiated sperms. As the duration of irradiation increased, the acrosome of sperms tended to suffer greater damage, until the sperms eventually lost their flagella. Abnormalities in these structures have appeared to account, at least in part, for the decline of the fertilization rate of eggs inseminated with UV-irradiated sperms.

Introduction

As a form of development in which eggs are activated by sperm which does not contribute genetically to the resulting embryo, artificial gynogenesis has been successfully induced in many fish species and used for examining sex-determination (1) and gene-centromere recombination (2), as well as production of inbred line, mono-sexual broods or clones (3-8).

In molluscs, induction of gynogenetic diploids has been recently reported for *Haliotis discus hannai* (9), *Crassostrea gigas* (10), *Mytilus edulis* (11) and *Mytilis galloprovincialis* (12). However, efficient procedures for induction of gynogenetic

diploid have not been established for molluscs as for fishes. Moreover, there has been no report on the production of adult gynogenetic molluscs. One of the plausible reasons for this is considered to be the decreasing fertilization rate with increasing UV-irradiation time of sperm that has been observed in all these studies. Kijima (13) proposed that UV irradiation affects not only the sperm genome, but also the acrosome structure of sperm of the Pacific abalone, but did not submit any direct evidence.

In the present study, the effect of UV irradition on sperm morphology was examined by scanning electron microscopy, and various durations of UV irradiation of sperm were also examined to determine the optimum conditions for efficient induction of gynogenesis in the Pacific abalone *H. discus hannai*.

Materials and Methods

Gamete collection

The Pacific abalone, *H. discus hannai* (shell length, 9.6 ± 0.7 cm; shell width, 6.7 ± 0.7), were obtained along the coast of Miyagi and Iwate Prefectures, and reared in running seawater. Spawning of gametes was artificially induced by the stimulations of dryness and UV-irradiated seawater. Discharged eggs were collected by suction and rinsed in filtered seawater several times. Sperm suspension was prepared at a concentrations of 4×10^6 sperm/ml by dilution with filtered seawater.

UV irradiation of sperm and insemination

2 ml of sperm suspension was spread on a 9.0 cm diameter plastic petri dish (Nunclon dish; Nalge Nunc Co., Denmark). The dish was placed on a recipro shaker (TAITEC Inc., Koshigaya, Japan) 15 cm below a 18 W UV germicidal light (TOSHIBA GL15, 254 nm; Toshiba Inc., Tokyo, Japan) that provided a UV intensity of 720 μW/cm^2/s as measured by a digital radiometer (DRC-100X; Spectronics Inc., Westbury, NY). The sperm was exposed to the UV light for either 0 (control), 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 60 s. During the UV irradiation, the sperm suspension was shaken at 40 cycles/min.

At completion of irradiation, 8 ml of egg suspension ($1\text{-}3 \times 10^3$ egg/ml) was added to each petri dish, mixed and finally transferred to a beaker for culture at room temperature (22–23°C). The rates of fertilization and larval normality were calculated by counting the cleaved eggs and normal veligers at 2 and 20 h after insemination, respectively. The experiment was repeated three times separately.

Ploidy determination

The ploidy of embryos was determined with chromosome preparations at 15 h postinsemination. Samples of the larvae (trochophores) were collected from

each group, placed in 0.1% colchicine for 2 h, subjected to a hypotonic treatment with 0.075 M KCl solution for 30 min, and fixed by Carnoy's solution (methanol : acetic acid, 3 : 1, v/v). After fixation, the samples were dropped on warmed glass slides, air-dried and stained with 2% Giemsa solution diluted with phosphate buffer (pH 6.8). Chromosome numbers were counted from well spread metaphase figures.

Scanning electron microscopy (SEM) of spermatozoa

The sperm suspension UV-irradiated for either 0, 10, 20, 60 or 120 s was placed on a small piece of slide glass (approximately 5×5 mm) coated with 0.1% poly-L-lysine (Wako Pure Chem. Ind., Ltd., Osaka, Japan), prefixed with 1% paraformaldehyde (Wako Pure Chem. Ind., Ltd.) and 2.5% glutaraldehyde (EM Sci. Inc., Fort Washington, Pennsylvania) in 0.2 M phosphate buffer (pH 7.4) for 2 h at room temperature, washed three times in phosphate buffered saline (PBS, pH 7.2, 0.85% NaCl), and then postfixed in 2% Osmic acid solution (Wako Pure Chem. Ind., Ltd.) for 1 h at 4°C. After washing three times with PBS, the samples were dehydrated in ethanol (50–100%), freeze-dried in *t*-butyl alcohol using an HITACHI ES2030 freeze dryer (Hitachi Inc., Japan), attached to larger metal discs, sputter-coated with Platinum-Paladium using an HITACHI E-1030 ion sputter, and observed with an HITACHI S-4200 scanning electron microscope.

Results

Effects of UV irradiation dosage on the rates of fertilization and larval normality

Relationships between UV irradiation duration and the rates of fertilization and normal development to veliger larvae are shown in Fig. 1. In experiments 1, 2 and 3, the fertilization rate for controls was more than 85%, but was apparently reduced with increasing exposure time, falling to 50–60% by 20 s and to approximately 30% by 40 s irradiation. As the irradiation duration increased, the rate of normal development to veligers decreased sharply, and became 0 by 5–10 s irradiation in each of the three experiments.

Chromosome observation

Mitotic metaphase plates from the larval cells of *H. discus hannai* are shown in Fig. 2. Figure 3 presents the frequency distribution of chromosome numbers in larval cells from 0 (control), 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 s irradiation groups in experiment 1. The modal number of the control group was 36 (Fig. 2A), the diploid number for *H. discus hannai* (14). For the 5 s irradiation group, the mode at N=36 was still verified, and aneuploid cells having chromosome numbers ranging from haploid to diploid number were observed (Fig. 2B). For the 10 and 15 s irradiation groups, in spite of a small number of cells showing the

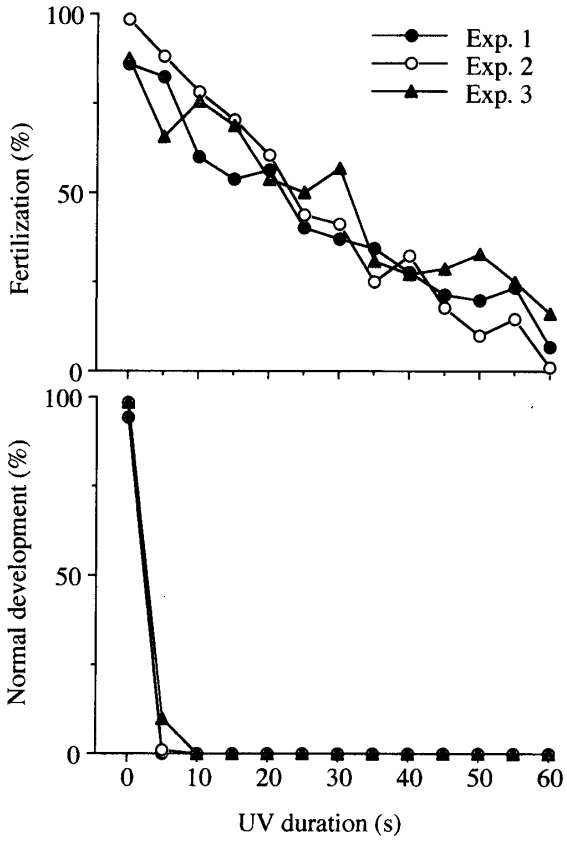

FIG. 1. Relationships between UV irradiation duration and the rates of fertilization and normal development to veliger larvae in the Pacific abalone, *Haliotis discus hannai*.

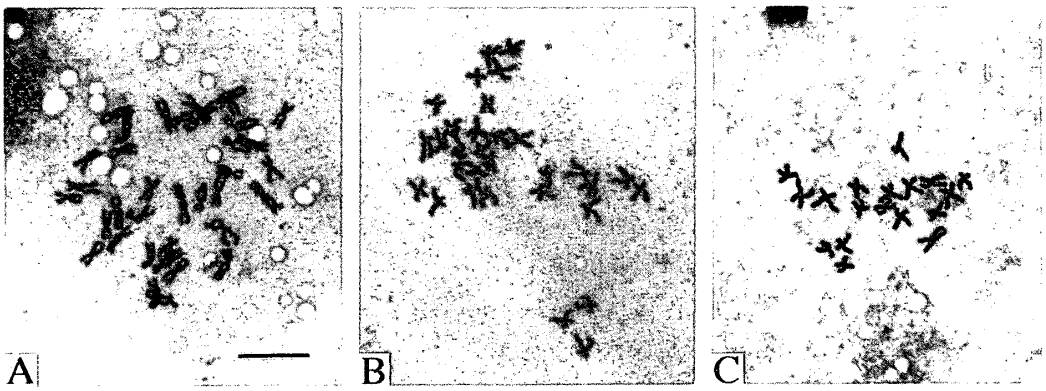

FIG. 2. Mitotic metaphase plates from the larval cells of *H. discus hannai*. A : a diploid cell with 36 chromosomes from the control group ; B : an aneuploid cell showing 31 chromosomes from the 5 s irradiation group ; C : a haploid cell with 18 chromosomes from the 10 s irradiation group. Scale bar = 10 μm.

diploid number, the mode at N = 18, the haploid number, was clearly observed (Fig. 2C). For the 20 s irradiation group, the mode at N = 18 became more distinct and no cells exhibited the diploid number. The mode at N = 18 remained

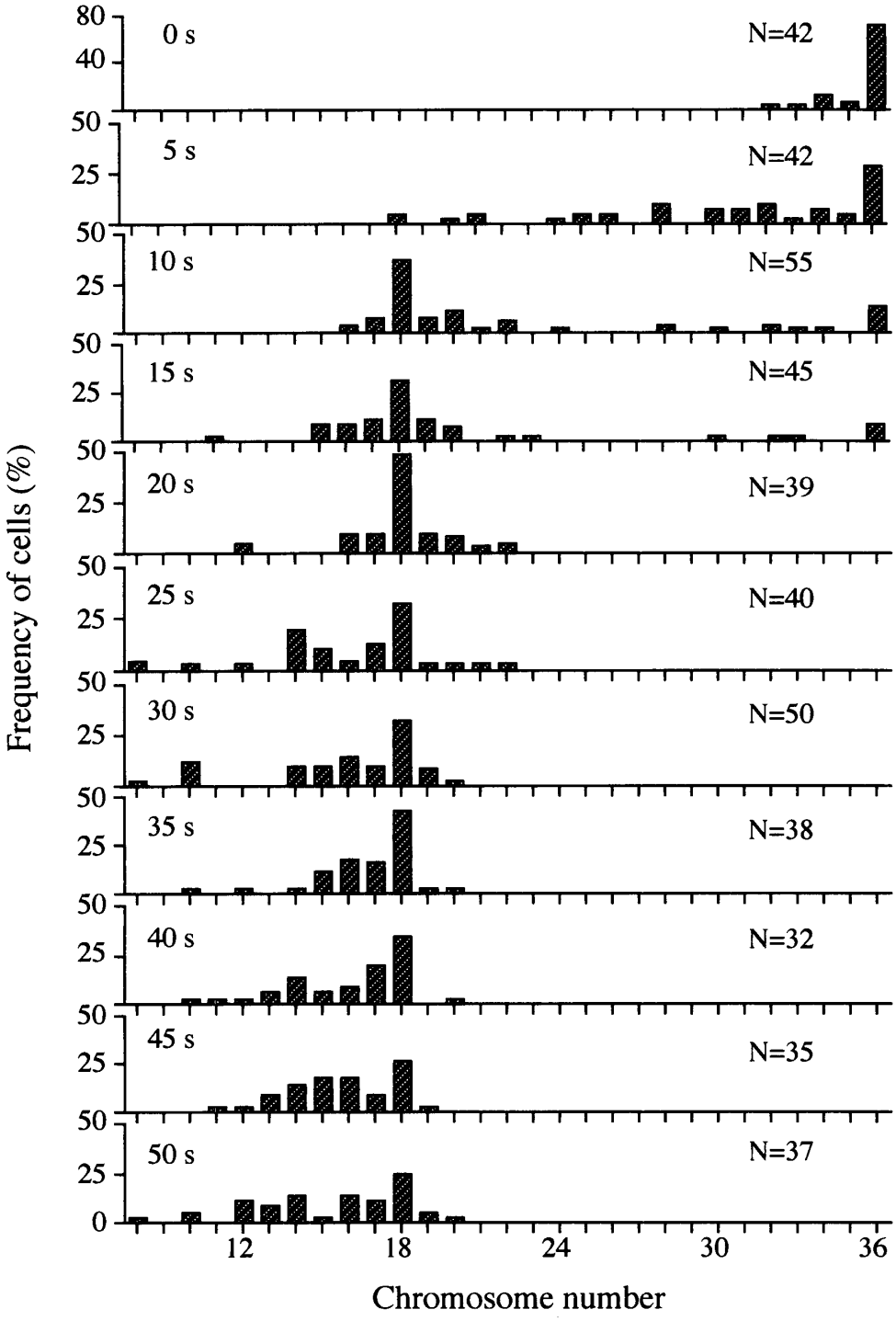

FIG. 3. Frequency distribution of chromosome numbers in larval cells of the *H. discus hannai* by various durations of UV irradiation in experiment 1. N indicates the total number of cells observed.

in the 25, 30, 35 and 40 s irradiation groups, but became less apparent in the 45 and 50 s irradiation groups. The results for experiments 2 and 3 were identical to those for experiment 1.

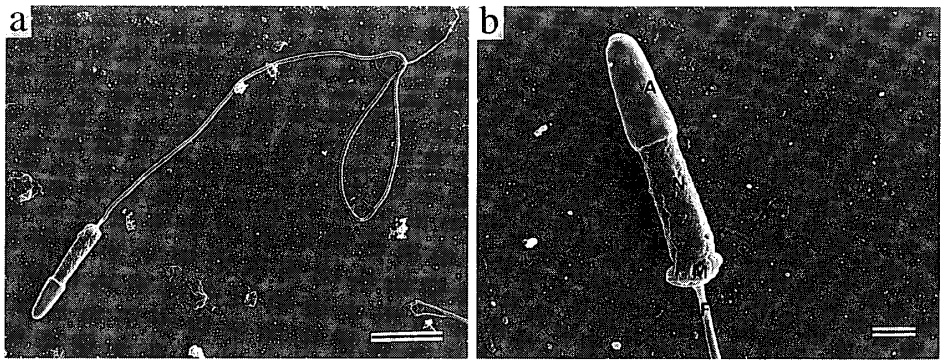

FIG. 4. SEM micrographs of normal spermatozoa of *H. discus hannai*. (a) a whole spermatozoon (bar = 4 μm); (b) the nucleus region of a spermatozoon (bar = 1 μm). N, Nucleus; A, Acrosome; M, Mitochondria; F, Flagellum.

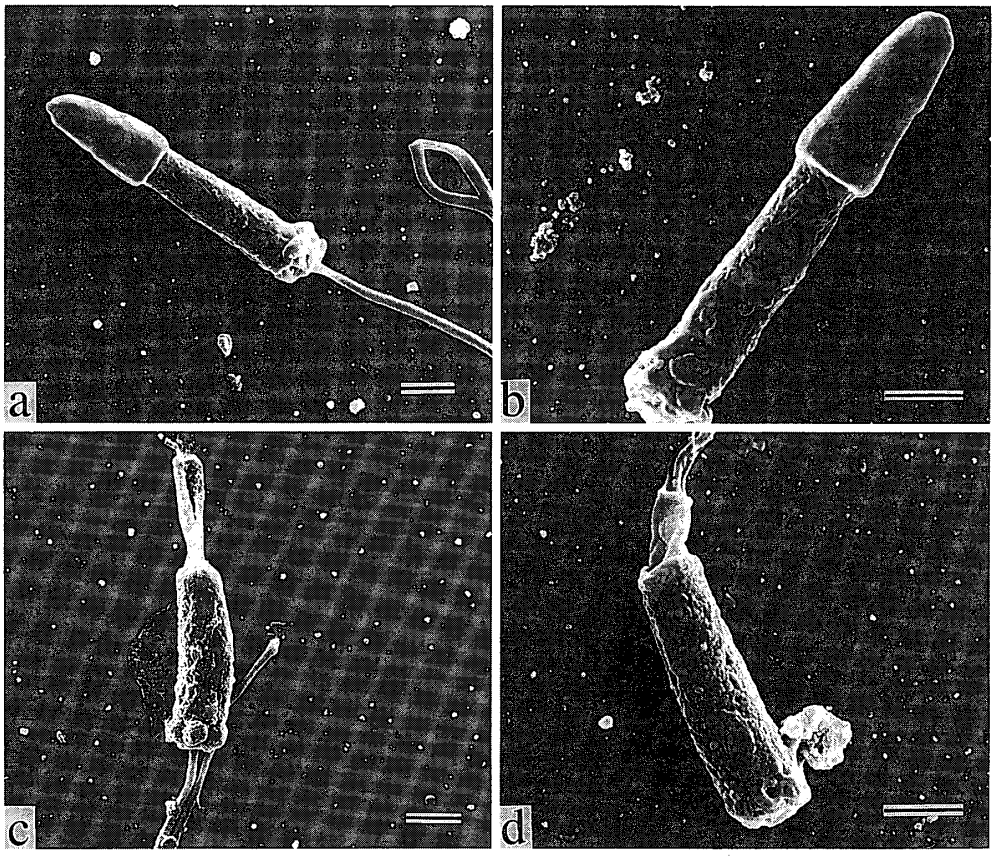

FIG.5. SEM micrographs of UV-irradiated spermatozoa (all bars = 1 μm). (a) a 10 s irradiated spermatozoon characteristic of group A, in which no morphological changes could be observed; (b) a 10 s irradiated spermatozoon characteristic of group B, in which the flagellum was lost; (c) a 20 s irradiated spermatozoon characteristic of group C, in which the acrosome was abnormal; (d) a 120 s irradiated spermatozoon characteristic of group D, in which the acrosome and flagellum are abnormal.

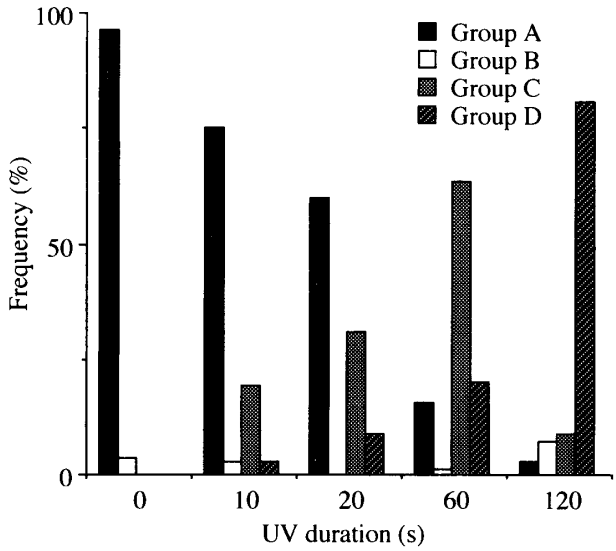

FIG. 6. Frequency distribution of four groups (A-D) of sperms in the various UV irradiation groups. The groups (A-D) correspond to those in Fig. 5.

Effect of UV irradiation on sperm morphology

The structure of normal untreated sperm was observed by SEM for comparison with the irradiated sperm. Spermatozoon consisted of three major parts: a bullet-shaped head, a short midpiece and a single flagellum (Fig. 4a). The head measured 5.9 ± 0.2 μm in length and 1.1 ± 0.0 μm in diameter ($n = 10$), and comprised the elongated nucleus and the conical acrosome (Fig. 4b). The midpiece showed the typical primitive arrangement with mitochondrial spheres around a centriolar apparatus.

Next, the sperms that had been irradiated for 0, 10, 20, 60 and 120 s were examined. Based on the changes in their morphology, the irradiated sperms were classified into the following four groups: Group A sperms showed no apparent change in morphology (Fig. 5a); Group B sperms had a missing flagellum (Fig. 5b); Group C sperms had an abnormal acrosome (Fig. 5c); and Group D sperms had both a missing flagellum and an abnormal acrosome (Fig. 5d). For each irradiation duration, more than fifty randomly selected sperms were used to classify the sperms into groups A-D. Figure 6 shows the changes in distribution of the four groups of sperms in different irradiation groups. The group A type accounted for 96.3% of the control group sperms, but this percentage decreased significantly with increasing exposure time. The percentage of group B sperms was consistently low and changed little in all groups. Group C sperms were not observed in the control group but occurred in all irradiation groups, accounting for 31.1% and 63.3% of sperms in the 20 s and 60 s irradiation groups, respectively. Group D sperms markedly increased with increasing irradiation duration, reaching a maximum proportion of 80.9% in the 120 s irradiation group.

Discussion

The present results agreed with those of the previous studies (13, 15), demonstrating that haploid gynogenesis of the Pacific abalone was successfully induced by 20-40 s UV irradiation of 720 μW/cm^2/s. Because a higher fertilization rate is necessary for efficient induction of gynogenesis, 20 s UV irradiation is considered to be the optimal dose for genetic inactivation of the abalone sperm. In *C. gigas*, the threshold dosage was 5-6 min at a UV intensity of 1,080 μW/cm^2/s (10). In *M. galloprovincialis*, 2 min irradiation (620 μW/cm^2/s) was the most successful in achieving gynogenetic development (12). The optimum dose for genetically inactivating sperm might vary from species to species.

As reported by Arai *et al.* (15) and Kijima (13), fertilization rate apparently declined with increasing irradiation duration in the Pacific abalone. This result indicates that the ability of sperm to activate eggs was reduced with increasing irradiation dose. Since this decrease in fertilization rate is not observed in gynogenetic induction of fish species (16-18), Kijima (13) proposed that not only the nuclear genome but also the acrosome structure (which is absent in teleost fishes) is damaged by UV irradiation in the abalone sperm.

In the present study, both the acrosome and flagellum were clearly destroyed in the UV-irradiated sperms. As the duration of UV irradiation increased, the acrosome of sperms tended to suffer greater damage, until eventually the sperms lost their flagella. A similar phenomenon has been observed in the Pacific oyster (19). Because the flagellum and acrosome are essential for the sperm to move to and penetrate the egg envelope and then fertilize the egg, abnormalities in these structures have appeared to account, at least in part, for the decline of the fertilization rate of eggs inseminated with UV-irradiated sperms. A small number of sperms in which only the flagellum was lost (Group B sperms) were retained in each treatment group, including the control group, and thus Group B sperms were considered to result from physical damage occurring during sample fixation rather than from the effects of UV irradiation.

In this study, haploid gynogenetic development was successfully induced in the Pacific abalone; however, the fate of the UV-irradiated sperm nucleus after its incorporation into the egg remains to be clarified. In *C. gigas*, the behavior of the UV-irradiated sperm nucleus has been examined by fluorescence microscopy, with the result that paternally derived genetical materials were suggested not to contribute to the next generation (20). Further studies on the mechanisms of gynogenetic induction can be expected to improve the production of gynogenetic Pacific abalones.

Acknowledgements

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References

1) Avtalion, R.R. and Don, J., Sex-determination genes in tilapia : a model of genetic recombination emerging from sex ratio results of three generations of diploid gynogenetic *Oreochromis aureus*. *J. Fish Biol.*, **37**, 167-173 (1990).
2) Arai, K., Fujino, K., Sei, N., Chiba, T. and Kawamura, M., Estimating rate of gene-centromere recombination at eleven isozyme loci in the *Salvelinus* species. *Nippon Suisan Gakkaishi*, **57**, 1043-1055 (1991).
3) Fujino, K., Historical aspects of research and development of genome manipulation technologies. In "*Chromosome Manipulation and its Application for Aquaculture*", Eds. Suzuki, R., Koseisha koseikaku, Tokyo, 9-20 (1989) (in Japanese).
4) Tabata, K. and Gorie, S., Induction of gynogenesis diploids in *Paralichthys olivaveus* by suppression of the 1st cleavage with special reference to their survival and growth. *Nippon Suisan Gakkaishi*, **54**, 1867-1872 (1988).
5) Taniguchi, N., Seki, S., Fukai, J. and Kijima, A., Induction of two types of gynogenetic diploids by hydrostatic pressure shock and verification by genetic marker in ayu. *Nippon Suisan Gakkaishi*, **54**, 1483-1491 (1988).
6) Fujioka, Y., Induction of gynogenetic diploids and cytological studies in honmoroko *Gnathopogon caurulescens*. *Nippon Suisan Gakkaishi*, **59**, 493-500 (1993).
7) Naruse, K., Ijiri, K., Shima, A. and Egami, N., The production of cloned fish in the medaka (*Oryzias latipes*). *J. Exp. Zool.*, **236**, 335-341 (1985).
8) Kobayashi, T., Ide, A., Hiasa, T., Fushiki, S. and Ueno, K., Production of cloned amago salmon *Oncorhynchus rhodurus*. *Fisheries Sci.*, **60**, 275-281 (1994).
9) Fujino, K., Arai, K., Iwadare, K., Yoshida, T. and Nakajima, S., Induction of gynogenetic diploid by inhibiting second meiosis in the Pacific abalone. *Nippon Suisan Gakkaishi*, **56**, 1755-1763 (1990).
10) Guo, X., Hershberger, W.K., Cooper, K. and Chew, K.K., Artificial gynogenesis with ultraviolet light-irradiated sperm in the Pacific oyster, *Crassostrea gigas*. I. Induction and survival. *Aquaculture*, **113**, 201-214 (1993).
11) Fairbrother, J.E., Viable gynogenetic diploid *Mytilus edulis* (L.) larvae produced by ultraviolet light irradiation and cytochalasin B shock.

Aquaculture, **126**, 25-34 (1994).

12) Scarpa, J., Komaru, A. and Wada, K.T., Gynogenetic induction in the mussel, *Mytilus galloprovincialis*. *Bull. Natl. Res. Inst. Aquaculture*, **23**, 33-41 (1994).

13) Kijima, A., Effect of irradiation on genetic inactivation of sperm using marketing tissue culture petri-dish in the Pacific abalone *Haliotis discus hannai*. *Tohoku J. Agri. Res.*, **42**, 73-81 (1992).

14) Arai, K., Tsubaki, H., Ishitani, Y. and Fujino, K., Chromosomes of *Haliotis discus hannai* Ino and *H. discus* Reeve. *Bull. Japan. Soc. Sci. Fish.*, **48**, 1689-1691 (1982).

15) Arai, K., Naito, F., Sasaki, H. and Fujino, K., Gynogenesis with ultraviolet ray irradiated sperm in the Pacific abalone. *Bull. Japan. Soc. Sci. Fish.*, **50**, 2019-2023 (1984).

16) Onozato, H. and Yamaha, E., Induction of gynogenesis with ultraviolet rays in four species of salmoniformes. *Nippon Suisan Gakkaishi*, **49**, 693-699 (1983) (in Japanese).

17) Taniguchi, N., Kijima, A., Fukai, J. and Inada, Y., Conditions to induce triploid and gynogenetic diploid in ayu *Plecoglossus altivelis*. *Nippon Suisan Gakkaishi*, **52**, 49-53 (1986) (in Japanese).

18) Tabata, K., Gorie, S. and Nakamura, K., Induction of gynogenetic diploid in hirame *Paralichthys olivaceus*. *Nippon Suisan Gakkaishi*, **52**, 1901-1904 (1986) (in Japanese).

19) Li, Q., Osada, M., Kashihara, M., Hirohashi, K. and Kijima, A., Effects of ultraviolet irradiation on genetical inactivation and morphological features of sperm of the Pacific oyster *Crassostrea* gigas. (submitted).

20) Li, Q., Osada, M., Kashihara, M., Hirohashi, K. and Kijima, A., Cytological observations on nuclear behavior in normal and gynogenetic eggs of the Pacific oyster *Crassostrea* gigas. (Submitted).