1132

Journal of Shellfish Research, Vol. 14, No. 2, 405–410, 1995.

ULTRASTRUCTURE OF SPERMATOZOA IN INDUCED TETRAPLOID MUSSEL *MYTILUS GALLOPROVINCIALIS* (LMK.)

AKIRA KOMARU,[1] JOHN SCARPA,[2] AND KATSUHIKO T. WADA[1]

[1]*National Research Institute of Aquaculture*
Fisheries Agency
Nansei, Mie
Japan 516–01
[2]*Harbor Branch Oceanographic Inst., Inc.*
Fort Pierce, Florida 34946

ABSTRACT Ultrastructure of spermatozoa in artificially induced tetraploid mussels, *Mytilus galloprovincialis*, was examined by light and electron microscopy and compared to that in normal diploid mussels. Sixteen tetraploid mussels were examined. Eight were found to be males, seven had spent or undeveloped gonads for which sex could not be determined, and one was an hermaphrodite. Ultrastructure of spermatozoa from tetraploids was similar to diploids, but there were some differences. Differences included the number of mitochondria (5 to 7 in tetraploids compared to only 5 in diploids) and the presence of abnormal acrosomes (2.4% in tetraploids, 0% in diploids). The mean size of spermatozoa in tetraploids was larger than that from diploids: acrosome length (4.2 μm vs. 3.0 μm), nuclear width (2.1 μm vs. 1.8 μm), nuclear length (2.1 μm vs. 1.9 μm), and flagellar length (73 μm vs. 63 μm). The DNA content of spermatozoa in tetraploids was about 2 times that of spermatozoa from diploids. Oocytes were observed in the hermaphroditic tetraploid, but only in very low numbers. These findings indicate spermatozoa will be capable of fertilization, and therefore, useful in the production of triploids by crossing to normal haploid oocytes.

KEY WORDS: *Mytilus galloprovincialis*, tetraploid, spermatozoa, ultrastructure

INTRODUCTION

Induced triploid bivalves are useful in aquaculture because the reduced gamete output improves meat quality and growth (Allen and Downing 1991, Akashige and Fushimi 1992). Triploidy is usually induced by preventing meiosis II of fertilized eggs. Several procedures (chemical treatment, hydropressure, and temperature shock) for the induction of triploidy in bivalves have been successful (Allen 1987, Beaumont and Fairbrother 1991). However, the survival of larvae in treated groups has been found to be lower than the controls (Tabarini 1984, Downing and Allen 1987, Wada et al. 1989, Utting and Child 1994). Additionally, the percentages of triploid induction were not always 100% for these studies.

A novel method to increase triploid induction rates and survival would be the use of multiple-genome gametes. The crossing of gametes from tetraploids and diploids has been shown to produce all triploid populations in brown frog (Kawamura et al. 1963) and rainbow trout (Chourrout et al. 1986). This indicates that the gametes from tetraploids contained a diploid genome complement.

In bivalves, viable spermatozoa with a DNA amount 1.5 times that of haploid spermatozoa were obtained from triploid Pacific oysters (Akashige 1990). Eggs fertilized with these sperm were aneuploid, containing 2.5 times the DNA level of haploid sperm (Akashige 1990). Oocytes from triploid Japanese pearl oysters fertilized with spermatozoa from diploids yielded some juveniles with either a diploid or triploid genome (Komaru and Wada 1993). This indicated that a small proportion of eggs contained twice the genome complement. Therefore, the use of "diploid gametes" from tetraploids for crossing with haploid gametes from diploids may be an efficient procedure for the production of triploids. We have previously reported the successful induction of tetraploidy in mussels (Scarpa et al. 1993). In the present study, gamete production and ultrastructure in tetraploid mussels were compared to that of diploid mussels by light and electron microscopy in order to delineate their possible performance characteristics.

MATERIALS AND METHODS

Organisms

The parental mussels (*Mytilus galloprovincialis* Lamarck) used to produce the tetraploid and diploid mussels examined in the present study were collected in January, 1993 from a natural population found in Gokasho Bay, Mie Prefecture, Japan (Scarpa et al. 1993). Mussels were spawned the same day as collection and tetraploidy was induced by inhibiting meiosis in fertilized eggs of mussels with cytochalasin B (Scarpa et al. 1993). After 3 months of controlled culture the spat were transferred to a pearl net and hung from a raft in Gokasho Bay.

Mussels were sampled in March and April, 1994. The mean shell height and standard deviation of mussels used were: tetraploid (3.13 ± 0.39 cm, n = 11), diploid (3.70 ± 2.06 cm, n = 8) in March and tetraploid (3.48 ± 0.58 cm, n = 5), diploid (4.90 and 3.80 cm, n = 2) in April. Large mussels were not sampled in this study as Scarpa et al. (1993) reported that tetraploids were only found in the smaller size grouping.

Histological Procedures

Gonadal tissue was fixed with 4% glutaraldehyde in cacodylate buffer (pH 7.5) (Komaru et al. 1994) for the March sample and Bouin fixative for the April sample. Tissue samples were then dehydrated through a graded ethanol series and embedded in paraffin. Sections (6 μm) were cut, placed on glass slides, and stained with acid-haemalaum and eosin.

Tissue samples fixed with 4% glutaraldehyde in cacodylate buffer were also prepared for electron microscopy following the procedure by Komaru et al. (1994). Small pieces (1–2 mm^2) of gonadal tissue were postfixed with 1% osmium tetroxide, dehydrated through a graded ethanol series and embedded in epoxy resin Quetol 812 (Nisshin E.M. Co., Tokyo, Japan). Ultrathin

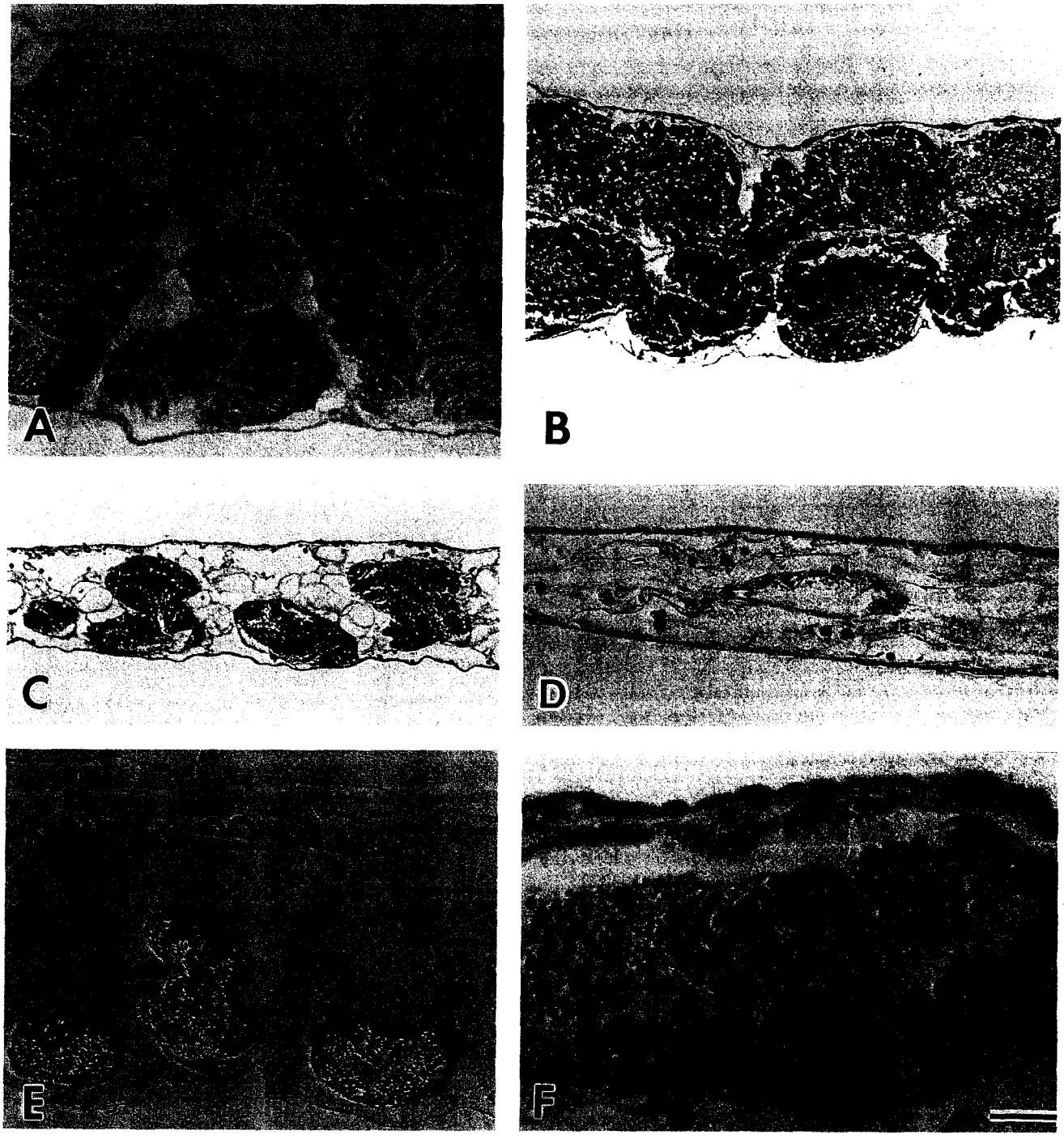

Figure 1. Light micrograph of gonadal tissue section from a diploid (A) and tetraploid (B–F) mussel, *Mytilus galloprovincialis*. A: Diploid mature testis. B,C: Tetraploid mature testis. D: Tetraploid spent testis. E,F: Tetraploid hermaphrodite gonad. Scale; A,B,C,E: 100 μm, D: 50 μm, F: 25 μm.

sections were stained with uranyl acetate and lead citrate, and observed using a JEOL 1200EX transmission electron microscope.

DNA Quantification

Ploidy of mussels was determined by DNA microfluorometry following the procedure of Komaru et al. (1988). Gill cells were dissociated from glutaraldehyde-fixed materials by mincing with scissors in 50% acetic acid, dropped on a prewarmed glass slide and air dried. The cells were stained with the DNA specific dye DAPI and the fluorescence intensity per nucleus (n = 30) was measured by microfluorometry. The DNA content of spermatozoa (n = 100) was also estimated using this procedure.

Spermatozoa Measurements

Nuclear width, nuclear length, and acrosome length were measured from the paraffin embedded material stained with acid-haemalaum and eosin.

Spermatozoa from gonads fixed with 4% glutaraldehyde were

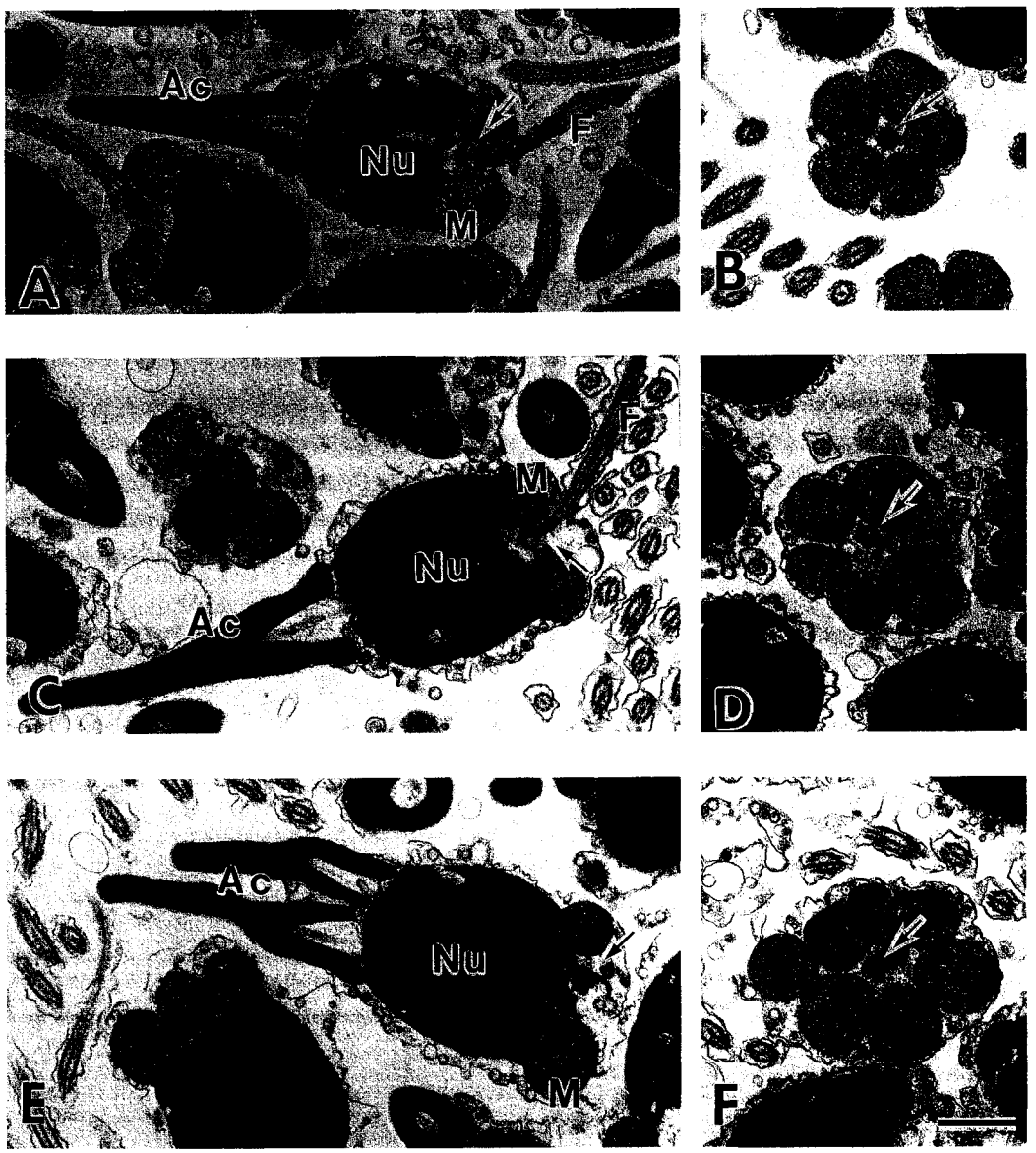

Figure 2. Electron micrograph of spermatozoa in diploid (A,B) and tetraploid (C–F) mussel, *Mytilus galloprovincialis*. A: Longitudinal section through spermatozoon in diploid. B: Transverse section through midpiece in diploid. C: Longitudinal section through spermatozoon in tetraploid. D,F: Transverse sections of through midpiece with 6 (D) and 7 (F) mitochondria (M) in tetraploids. E: Longitudinal section through abnormal spermatozoon with 2 acrosomes. Acrosome = Ac, Nucleus = Nu, Flagellum = F. Arrows indicate centrioles. Scale: 1 μm.

dispersed by mincing with scissors in 50% acetic acid, dropped on a prewarmed glass slide and air dried. Unstained preparations of dispersed spermatozoa were observed by phase contrast microscopy. Flagella length was measured with an ocular micrometer. The percentage of spermatozoa with 2 acrosomes was also estimated. The mean size of spermatozoa components (n = 30) between tetraploids and diploids was statistically analyzed using Student's t-test (Sakuma 1964).

The number of mitochondria per spermatozoa were counted on TEM negatives showing transverse sections of the midpiece.

RESULTS

Gamete Production

In the March sample, 8 diploids and 11 tetraploids were examined. Among the diploids, three were mature males (Fig. 1A), one was a mature female, and four had spent gonads. Among tetraploids, four contained spermatozoa (Fig. 1B,C) but were less prolific compared to the diploids (Fig. 1A). The other 7 tetraploids had spent gonads, but two of these seven were identified as male because of residual spermatozoa (Fig. 1D). In the April sample, one of 5 tetraploids was an hermaphrodite, two were identified as male because of residual spermatozoa, and the other two had spent gonads.

In the hermaphrodite tetraploid, oocytes and spermatozoa were usually observed in different acini (Fig. 1E). Acini filled with spermatozoa predominated, but spermatozoa and oocytes were observed occasionally in the same acini (Fig. 1F). The oocytes had a large nucleus, prominent nucleoli, and well developed cytoplasm, although the number of oocytes were few. The means ± sd of the long and short axis of 10 oocytes from the hermaphrodite were 68.5 ± 4.0 μm and 52.6 ± 7.2 μm, respectively. In com-

TABLE 1.

Size (mean ± standard deviation) of spermatozoa components from 2 tetraploid and 1 diploid mussel in March, 1994.

	Tetraploid		
	No. 1	No. 2	Diploid
Length of Acrosome (μm)	4.27 ± 0.30*	4.22 ± 0.22*	2.98 ± 0.08
Width of Nucleus (μm)	2.16 ± 0.06*	2.14 ± 0.06*	1.78 ± 0.07
Length of Nucleus (μm)	2.06 ± 0.06*	2.04 ± 0.05*	1.85 ± 0.06
Length of Flagellum (μm)	72.3 ± 2.3*	75.1 ± 2.3*	63.0 ± 1.9

* Significantly different ($P < 0.01$). Number of sperm measured was as follows; tetraploid: 30, diploid: 30.

parison, the means of the long and short axis of 10 oocytes in one mature diploid were 60.1 ± 2.6 μm and 45.2 ± 4.4 μm, respectively.

Ultrastructure of Spermatozoa

General ultrastructural morphology of spermatozoa produced by tetraploids (Fig. 2C,D) was almost identical to that of spermatozoa produced by diploids (Fig. 2A,B), except for the number of mitochondria and increased size of flagellum, acrosome, and nucleus (Table 1). Spermatozoa produced by tetraploids (Fig. 2C,D) were composed of an elongated acrosome (4.2 μm), an electron dense nucleus (2.2 μm × 2.1 μm), spheroid mitochondria and a flagellum (74 μm). These measured parameters were all significantly larger ($P < 0.01$) in the tetraploids compared to the diploids.

The acrosome was elongated and conical, composed of an outer layer with electron dense material and inner layer of electron lucent material. The nucleus was filled with fine electron dense materials and was barrel shaped. As shown in Table 2 the number of mitochondria per spermatozoa produced by tetraploids ranged from five to seven (Fig. 2D,F) with a modal number of six, while the number of mitochondria in diploids was always five (Fig. 2B). Centrioles were observed in the mitochondrial ring (Fig. 2C,D) of tetraploids.

In testes from tetraploid most spermatozoa were not grossly different from those in diploids (Fig. 2C), but abnormal spermatozoa with 2 acrosomes were found occasionally (Fig. 2E). The percentage of spermatozoa with 2 acrosomes was 1.7% and 3.0% in 2 different tetraploids examined in the March sample (Table 3).

Figure 3 shows the DNA content of spermatozoa from tetraploids and diploids. Spermatozoa could be differentiated from other cell types because of the elongated nucleus, ensuring that only spermatozoa DNA levels were measured. The mean DNA content of spermatozoa from 2 tetraploids was about 2 times (2.10 and 1.95) that of spermatozoa from diploids.

TABLE 2.

Frequency distribution of the number of mitochondria in spermatozoa observed in tetraploid and diploid mussel in March, 1994.

Number of Mitochondria	Tetraploid (%)	Diploid (%)
4	0	0
5	25	100
6	50	0
7	25	0

Number of sperm observed was as follows; tetraploid: 20, diploid: 11.

TABLE 3.

The percentages of spermatozoa with abnormal acrosome produced by 2 tetraploid (No. 1, No. 2) and 2 diploid (No. 1, No. 2) mussels fixed in March, 1994.

	Tetraploid		Diploid	
	No. 1	No. 2	No. 1	No. 2
Abnormal Acrosome (%)	1.7	3.0	0	0
Normal Acrosome (%)	98.3	97.0	100	100
Number of Sperm Observed	423	427	328	253

DISCUSSION

Although gamete production and ultrastructure have been examined in a number of bivalves (Popham 1979, Hodgson and Bernard 1986, Dorange and Le Pennec 1989, Thielly et al. 1993), it is only recently that gamete ultrastructure in a polyploid bivalve has been reported (Komaru et al. 1994). In the present study, male tetraploids predominated in the sample taken; therefore, only spermatozoa ultrastructure could be observed and compared in detail.

Spermatozoa produced by tetraploid mussels were composed of an acrosome, nucleus, mitochondria, and flagellum and their morphology was almost identical to that of diploid mussels reported (Hodgson and Bernard 1986). Judging from ultrastructure, sperm from tetraploid mussels should be functional. Chourrout et al. (1986) reported that in trout the low fertilizing ability of spermatozoa produced by tetraploids was due to the difficulty of sperm penetrating the micropyle. In mussels this should not be of consequence since mussel sperm enter into the egg cytoplasm follow-

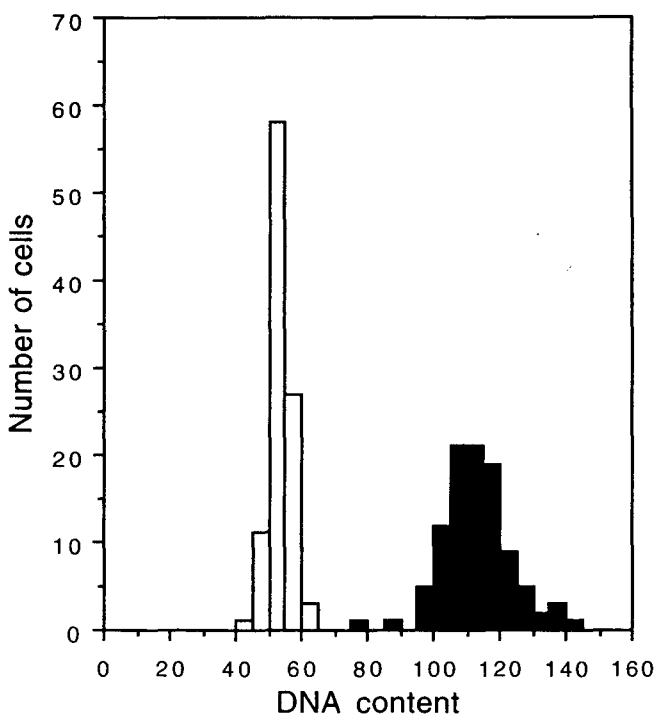

Figure 3. Relative DNA content of spermatozoa as measured by microfluorometry from diploid (white bars, n = 100) and tetraploid (black bars, n = 100) mussel *Mytilus galloprovincialis* in March 1994.

ing an acrosome reaction with the egg membrane (Niijima and Dan 1965). It should be examined whether the acrosome reaction of sperm from tetraploid mussels would be adequate and normal for penetration.

In all measured parameters, the size of spermatozoa in tetraploids was larger than that of diploids. The nuclear size may be increased due to the increased DNA content as shown by DNA microfluorometry. Flagella length and acrosome length in tetraploids were significantly larger. How the increased DNA content may give rise to a larger acrosome and longer flagellum is not apparent from our study.

It was interesting to observe that the number of mitochondria in spermatozoa from tetraploids was greater by one to two as compared to spermatozoa of diploids. In diploids, the number of mitochondria was five, as previously reported (Hodgson and Bernard 1986). It has been previously suggested that motility of spermatozoa produced by triploid Pacific oysters may be reduced, because the head was significantly larger (compared to spermatozoa from diploids) and the number of mitochondria was the same (Komaru et al. 1994). Sperm from tetraploids with only 5 mitochondria, which is the number found in sperm from diploids, may have reduced motility compared to the sperm of diploids. However, the increased number of mitochondria in the majority of sperm from tetraploids may negate the size increase with resultant motility similar to sperm from diploids.

Although differences of spermatozoa ultrastructure between tetraploids and diploids were primarily size related, another difference was the production of abnormal spermatozoa with 2 acrosomes. The spermatozoa produced by tetraploids most likely have 2 sets of chromosomes and the formation of 2 acrosomes may be related to this. The final morphogenetic process may sometimes be abnormal due to 2 sets of genes being expressed that regulate acrosome formation.

Spermatozoa produced by tetraploids will be useful for producing triploid and other polyploid levels if they contain 2 euploid chromosome sets. In the present study this was indicated indirectly by DNA microfluorometry. The induction of triploidy is usually achieved by preventing polar body formation in fertilized eggs from diploids (Allen 1987, Beaumont and Fairbrother 1991). Treatments used to induce triploidy generally reduce larval survival compared to untreated eggs and the proportion of triploid organisms is often less than 100% (Tabarini 1984, Downing and Allen 1987, Wada et al. 1989, Scarpa et al. 1994, Utting and Child 1994). If spermatozoa with a diploid genome were available the production of 100% triploid population could be easily achieved by inseminating normal haploid eggs with these "diploid sperm". Recently, this has been achieved in the Pacific oyster (Dr. Ximing Guo, pers. comm.). Additionally, if second polar body formation was inhibited in such a cross tetraploidy could theoretically be induced (Kawamura et al. 1963, Chourrout et al. 1986). A similar technique was used to produce tetraploids by inhibiting polar body I formation in eggs from triploid oysters fertilized with normal haploid sperm (Guo and Allen 1994).

In the present study, well-developed oocytes in an hermaphrodite tetraploid were also observed. A cross between oocytes and spermatozoa produced by tetraploids would also yield tetraploids if the oocytes had a diploid genome. Oocytes containing 2 genomes have been produced by triploid pearl oysters as indicated by the ploidy of surviving juveniles (Komaru and Wada 1994). "Diploid sperm" may be useful not only for the induction of an all triploid or polyploid population but for producing androgenetic organisms by crossing diploid sperm and genetically inactivated oocytes (Kusunoki et al. 1994).

ACKNOWLEDGMENT

Support for J. Scarpa was provided by the Science and Technology Agency of Japan and National Science Foundation (USA) under grant No. 91–01762. The authors wish to thank the reviewers for their helpful suggestions with this manuscript.

LITERATURE CITED

Akashige, S. 1990. Growth and reproduction of triploid Japanese oyster in Hiroshima Bay. pp. 461–468. *In:* M. Hoshi & O. Yamashita (eds.). *Advances in Invertebrate Reproduction 5*. Elsevier Science Publishers B.V., Amsterdam.

Akashige, S. & T. Fushimi. 1992. Growth, survival, and glycogen content of triploid Pacific oyster *Crassostrea gigas* in the waters of Hiroshima, Japan. *Nippon Suisan Gakkaishi* 58:1063–1071. (in Japanese with English abstract).

Allen, S. K., Jr. 1987. Genetic manipulations—critical review of methods and performances for shellfish, pp. 127–143. *In:* K. Tiwes (ed.). *Selection, Hybridization and Genetic Engineering in Aquaculture Vol. 2*. Heenemann GmbH & Co, Berlin.

Allen, S. K., Jr. & S. L. Downing. 1991. Consumers and "experts" alike prefer the taste of sterile triploid over gravid diploid Pacific oysters (*Crassostrea gigas*, Thunburg, 1793). *J. Shellfish Res.* 10:19–22.

Beaumont A. R. & J. E. Fairbrother. 1991. Ploidy manipulation in molluscan shellfish: a review. *J. Shellfish. Res.* 10:1–18.

Chourrout, D., B. Chevassus, F. Kreig, A. Happe, G. Burger & P. Renard. 1986. Production of second generation triploid and tetraploid rainbow trout by mating tetraploid males and diploid females—Potential of tetraploid fish. *Theor. Appl. Genet.* 72:193–206.

Dorange, G. & M. Le Pennec. 1989. Ultrastructural characteristics of spermatogenesis in *Pecten maximus* (Mollusca, Bivalvia). *Invert. Reprod. Dev.* 15:109–117.

Downing, S. L. & S. K. Allen, Jr. 1987. Induced triploidy in the Pacific oyster, *Crassostrea gigas:* optimal treatments with cytochalasin B depend on temperature. *Aquaculture* 61:1–15.

Guo, X. & S. K. Allen, Jr. 1994. Viable tetraploids in the Pacific oyster (*Crassostrea gigas* Thurnberg) produced by inhibiting polar body 1 in eggs from triploids. *Mol. Mar. Biol. Biotech.* 3:42–50.

Hodgson, A. N. & R. T. F. Bernard. 1986. Ultrastructure of the spermatogenesis of three species of Mytilidae (Mollusca, Bivalvia). *Gamete Res.* 15:123–135.

Kawamura, T., M. Nishioka & Y. Myorei. 1963. Reproductive capacity of autotetraploid males in brown frogs. *J. Sci. Hiroshima Univ.* Ser. B. Div. 1. 21:15–24.

Komaru, A. & K. T. Wada. 1993. Meiotic maturation and progeny of oocytes from triploid Japanese pearl oyster (*Pinctada fucata martensii*) fertilized with spermatozoa from diploids. *Aquaculture* 120:61–70.

Komaru, A., K. Konishi & K. T. Wada. 1994. Ultrastructure of spermatozoa from induced triploid Pacific oyster, *Crassostrea gigas*. *Aquaculture* 123:217–222.

Komaru, A., Y. Uchimura, H. Ieyama & K. T. Wada. 1988. Detection of induced triploid scallop, *Chlamys nobilis*, by DNA microfluorometry with DAPI staining. *Aquaculture* 69:201–209.

Kusunoki, T., K. Arai & R. Suzuki. 1994. Production of viable gynogens without chromosome duplication in the spinous loach *Cobitis biwae*. *Aquaculture* 119:11–23.

Niijima L. & J. Dan. 1965. The acrosome reaction in *Mytilus edulis* II. *J. Cell Biol.* 25:249–259.

Popham, J. D. 1979. Comparative spermatozoon morphology and bivalve phylogeny. *Malacol. Rev.* 12:1–20.

Sakuma, A. 1964. Bioassay—Design and analysis. University of Tokyo Press, Tokyo. 309 pp. (in Japanese).

Scarpa, J., K. T. Wada & A. Komaru. 1993. Induction of tetraploidy in mussels by suppression of polar body formation. *Nippon Suisan Gakkaishi* 59:2017–2023.

Scarpa, J., J. E. Toro & K. T. Wada. 1994. Direct comparison of six methods to induce triploidy in bivalves. *Aquaculture* 119:119–133.

Tabarini, C. L. 1984. Induced triploidy in the bay scallop, *Argopecten irradians*, and its effect on growth and gametogenesis. *Aquaculture* 42:151–160.

Thielly, M., M. Weppe & C. Herbaut. 1993. Ultrastructural study of gametogenesis in the French Polynesian Black pearl oyster *Pinctada margaritifera* (Mollusca, Bivalvia). I—Spermatogenesis. *J. Shellfish Res.* 12:41–47.

Utting, S. D. & A. R. Child. 1994. Genetic manipulation of the Manila clam (*Tapes philippinarum*) using cytochalasin B to induce triploidy. *Aquaculture* 120:271–282.

Wada, K. T., A. Komaru & Y. Uchimura. 1989. Triploid production in the Japanese pearl oyster, *Pinctada fucata martensii*. *Aquaculture* 76:11–19.