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Milt Properties of Four Flatfish Species and Fine Structure of Their Cryopreserved Spermatozoa

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The physico-chemical properties of fresh milt of marbled sole, *Limanda yokohamae*, brown sole, *Limanda herzensteini*, starry flounder, *Platichthys stellatus* and olive flounder, *Paralichthys olivaceus* among flatfishes, and the fine structure of their cryopresserved spermatozoa were investigated. The highest concentration of sperm among these four species was $3.60 \pm 1.35 \times 10^{10}$/mL in marbled sole. Osmolality and pH of seminal plasma in four flatfish species were approximately 330 mOsm/kg and 7.6~8.1, respectively. Seminal plasma compositions showed interspecific differences. The sperm heads of marbled sole, brown sole and starry flounder were ellipsoidal and that of olive flounder was round. The numbers of mitochondria of these four species were eight in marbled sole, seven in brown sole and starry flounder, and six in olive flounder. Cross-sectional view of flagellum showed typical 9+2 structure in all species. Most of sperms cryopreserved with a proper method had no visible ultrastructural changes after freeze-thawing, compared with the fresh sperm, but in a few cases, swelling of their heads and midpiece regions were observed.

Key words: Flatfish, Marbled sole, Brown sole, Starry flounder, Olive flounder, *Limanda yokohamae*, *Limanda herzensteini*, *Platichthys stellatus*, *Paralichthys olivaceus*, Milt property, Sperm cryopreservation, Spermatozoa morphology

Introduction

Flatfish Pleuronectiformes are the important and valuable species in the East Sea of Korea. Because of the failure of natural reproduction as a result of over fishing, artificial manipulation of flatfish reproduction is required to maintain the population. However, only limited information on physiology and biochemistry of sperm required for artificial reproduction is available for flatfish species. More fundamental data are needed to establish the criteria of milt quality, to study sex manipulation and to investigate the best condition for storing sperm of flatfish.

In mammals, the reproductive ability of males has been evaluated by chemical and physical examinations of milt. In fish, there have been few chemical or physical criteria to judge its reproductive ability. Several studies have described milt characteristics such as sperm density, motility and the composition of the seminal plasma (Hwang and Idler, 1969; Piironen and Hyvarinen, 1983; Stoss, 1983; Chang et al., 1995; 1998; 1999). Because milt quality influences the ability to fertilize eggs, it is one of the important factors to determine the success of seedling production in fish. In addition, the knowledge of milt characteristics is essential for developing a new diluent in the preservation of fish sperm.

Fine structure of sperm has been studied in 280 fish species (Mattei, 1991). The differences of sperm structure between externally and internally fertilizing teleosts were observed. While the sperm of externally fertilizing fish generally have spherical or ovoid head and a small midpiece, those of internally fertilizing fish have elongated heads and more complicated midpiece structure (Jamieson, 1991). The information on ultrastructure of spermatozoa is

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needed for understanding the spermatology, which may be valuable in developing a cryopreservation method of sperm.

Cryopreservation of the sperm of many teleosts has been well studied (Baynes and Scott, 1987; Cabrita et al., 1998; Drokin et al., 1998; Kerby et al., 1985; Lahnsteiner et al., 1996a; 1996b; 1996c; Linhart et al., 1993; Stoss and Holtz, 1981a; 1981b). Sperm damage is known to occur during cryopreservation by several reports (Billard, 1983; Gwo et al., 1993; Gwo and Arnold, 1992; Lahnsteiner et al., 1992; Chang et al., 1998). However, until now there have been hardly studied on cryopreservation and subsequent morphological damage in flatfish sperm, although evaluation of damage for cryopreserved sperm should be performed in developing optimal cryopreservation methods.

The objectives of this study were to investigate the physico-chemical properties of milt for evaluating its quality, and to compare the ultrastructure of fresh and cryopreserved sperm of four species of flatfish.

Materials and Methods

Fish specimens and milt collection

Mature and spermiating males of marbled sole, *Limanda yokohamae*, brown sole, *Limanda herzensteini* and olive flounder, *Paralichthys olivaceus* (Table 1) were captured near Kangnung waters, Korea during their spawning season (January to March in marbled sole, March to May in brown sole, and May and June in olive flounder). After transporting the males to Kangnung Marine Hatchery of National Fisheries Research and Development Institute (NFRDI), fish was kept in indoor 2 m^3 tanks supplied with sand-filtered seawater. Mature starry flounders were obtained from Uljin Marine Hatchery of NFRDI. In the case of starry flounder, *Platichthys stellatus*, luteinizing hormone releasing hormone analogue [LHRH-a; (pGlu-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt, Sigma)] pellets were implanted in the dorsal muscle to increase the milt volume.

Table 1. Measurements of male marbled sole, brown sole, starry flounder and olive flounder for milt collection

Species	Specimens	Total length (cm)	Body weight (g)
Marbled sole	26	26.7 ± 3.1	270.8 ± 93.3
Brown sole	81	21.0 ± 1.8	99.2 ± 29.3
Starry flounder	23	34.2 ± 1.5	574.0 ± 96.0
Olive flounder	24	33.1 ± 1.9	382.1 ± 71.9

Mean ± SD.

Prior to handling each specimen, it was first anesthetized with 3-aminobenzoic acid ethyl ester (MS-222). The urinary bladder of fish was gently emptied and the genital area was wiped with paper towel before milt was stripped by hand. Caution was taken to prevent a contamination of milt with urine or fecal materials. The milt collected from each fish was pooled and stored for a maximum of 1 hour on crushed ices before the onset of experiments. Only the sperm showing the vigorous movement of more than 90% progressive motility by microscopic observation were used in the following experiments.

Milt properties

The volume of the collected milt was measured by 1.5 mL Eppendorf tube. Sperm concentration was counted with a hemocytometer chamber under a microscope (×400) after 2,000×dilution with 2% eosin solution. Spermatocrit was determined after centrifugation at 12,000 rpm for 10 min in 75 mm capillary tubes (Bouck and Jacobson, 1976). Seminal plasma was collected after centrifugation of milt at 12,000 rpm for 10 min and stored at −80℃ until use. The pH and osmolality of the seminal plasma were measured with pH meter (pH/Ion Meter EP-880) and osmometer (The Advanced TM Osmometer), respectively. The concentrations of K^+, Na^+ and Cl^- and the contents of Ca, Mg and glucose in the seminal plasma were analyzed by VITROS DT II Chemistry system.

Milt cryopreservation procedure

The general freezing protocol followed the methods of Lahnsteiner et al. (1992). Briefly, milt of each species was diluted in the ice-cold diluent (2 g $NaHCO_3$; 1 g glucose; 0.4 g KCl; 7.5 g NaCl/1,000 mL distilled water) containing cryoprotectant (15% ethylene glycol in marbled sole; 10% dimethyl sulfoxide in brown sole and starry flounder; 10% ethylene glycol in olive flounder) in a ratio of 1:5 (milt:diluent), sucked into 0.5 mL straws and frozen with liquid nitrogen vapor in an insulated box

within 5 min of dilution. The straws were layed horizontally on a tray above 7 cm from the surface of liquid nitrogen. After the freezing period of 5 min, the straws were transferred into liquid nitrogen and stored for 10 days. For thawing, the straws were immersed in a 30℃ water bath for 20 sec. Thereafter, the straws removed from the water bath were cut off the plug and the thawed milt poured into vials for immediate examinations.

Morphological investigation

To investigate the ultrastructure of fresh and cryopreserved sperm, transmission electron microscope samples were prepared. The fresh or freeze-thawed milts were pre-fixed for 2 hour at 4℃ in 2.5% glutaraldehyde solution buffered by 0.1 M phosphate buffer solution (PBS, pH 7.2). After washing with PBS for 10 min, the samples were post-fixed in 1% osmium tetroxide (OsO_4) for 2 hour at 4℃. The samples were washed again with PBS, then serially dehydrated with ethanol from 50% to 100% and embedded in Epon 812. 0.5 μm-thick sections were first cut with ultramicrotome (LKB, Nova, Sweden) and then stained with toluidine blue to determine an investigation region. After that, 70 nm-thick sections (ultrathin section) were cut again. The sections were doublestained with uranylacetate and lead citrate solution and examined with transmission electron microscope (JEM 1200 E-XII, 60~80 Kv, JEOL, Japan).

Results

Milt properties

The physical properties of each milt from marbled sole, brown sole, starry flounder and olive flounder are shown in Table 2.

Marbled sole produced greater quantities of milt than other species. Sperm concentration and spermatocrit were the highest in marbled sole, which were $3.60 \pm 1.35 \times 10^{10}$/mL and 91.8 ± 7.4, respectively. The lowest values were in starry flounder, which were $0.87 \pm 0.33 \times 10^{10}$/mL and 51.6 ± 15.6, respectively.

Table 3 showed chemical properties of milt in four species. K^+ concentration was the highest value of 20.8 ± 9.0 mM/L in olive flounder, and the lowest value of 4.0 ± 1.0 mM/L in marbled sole. Na^+ con-

Table 2. Physical properties of milt of marbled sole, brown sole, starry flounder and olive flounder

Property	Marbled sole	Brown sole	Starry flounder	Olive flounder
Milt volume (mL/100 g BW)	1.7±1.5	0.5±0.4	0.4±0.2	0.4±0.3
Sperm conc. ($\times 10^{10}$/mL)	3.60±1.35	1.47±0.57	0.87±0.33	1.62±0.59
Spermatocrit	91.8±7.4	63.2±16.9	51.6±15.6	60.2±16.6

Mean ± SD (n=15).

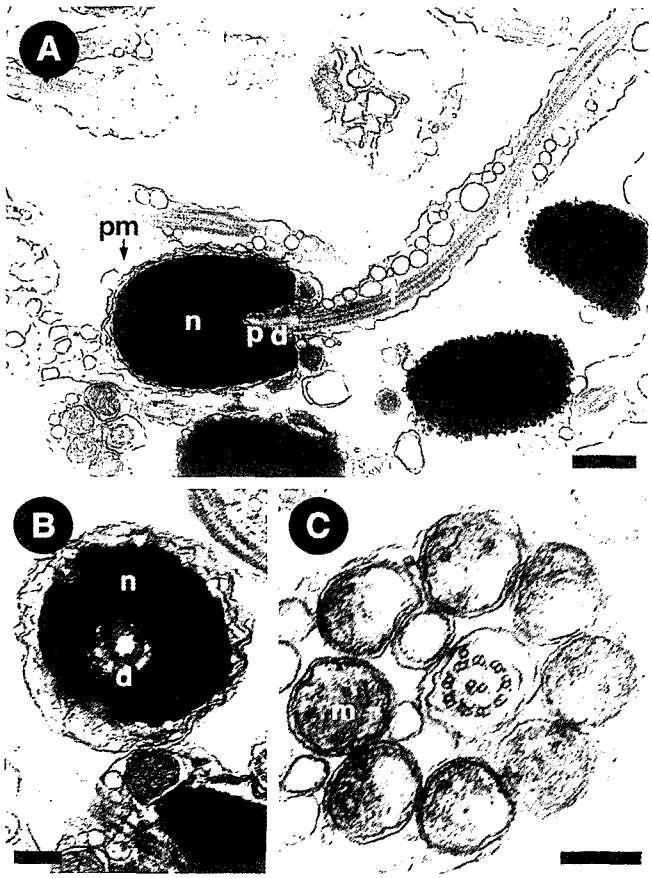

Fig. 1. Photographs of fresh spermatozoon of marbled sole by transmission electron microscope. A: Sagittal section of a spermatozoon. Note the head with the compact chromatin and centriole. Bar=0.5 μm. B: Cross section of the head having a distal centriole. Bar=0.2 μm. C: Cross section of midpiece with eight mitochondria and 9+2 structure of flagellum. Bar=0.2 μm. d: distal centriole, f: flagellum, m: mitochondrion, n: nucleus, p: proximal centriole, pm: plasma membrane.

Table 3. Chemical properties of seminal plasma in marbled sole, brown sole, starry flounder and olive flounder

Property	Marbled sole	Brown sole	Starry flounder	Olive flounder
K^+ (mmol/L)	4.0±1.0	10.8±4.1	7.5±3.2	20.8±9.0
Na^+ (mmol/L)	144.4±9.7	117.9±16.7	141.7±16.6	111.5±11.6
Cl^- (mmol/L)	124.9±6.5	116.7±16.5	117.4±11.9	118.8±9.7
Ca (mg/100 mL)	7.4±2.9	8.0±1.9	7.9±1.3	7.2±1.4
Mg (mg/100 mL)	30.8±18.0	48.0±15.9	2.4±1.1	54.0±10.1
Glucose (mg/100 mL)	25.5±4.7	24.3±2.6	31.0±3.9	23.8±6.2
pH	7.7±0.3	8.1±0.3	7.6±0.4	7.9±0.2
Osmolality (mOsm/kg)	336.4±22.0	337.3±22.4	328.5±20.3	334.3±26.6

Mean ± SD (n=15).

centrations in marbled sole and starry flounder showed higher values of 144.4 ± 9.7 mM/L and 141.7 ± 16.6 mM/L, respectively, than those of 117.90 ± 16.66 mM/L in brown sole and 111.5 ± 11.63 mM/L in olive flounder. Cl^-, calcium and glucose contents of four species were similar to each other. Osmolality and pH of seminal plasma of four species were within the ranges of 328.5~337.3 mOsm/kg and 7.6~8.1, respectively.

Fine structure of fresh spermatozoa

All of the fresh spermatozoa in four flatfish species consisted of three distinct parts of head, midpiece and tail. A plasma membrane tightly covered the head, midpiece and tail (Figs. 1, 2, 3 and 4). The heads of marbled sole, brown sole and starry flounder were ellipsoidal and that of olive flounder was round. The lengths and widths of heads in four species were 1.30~1.73 μm (1.53 ± 0.18 μm) and 0.85 ~1.22 μm (1.05 ± 0.16 μm) in marbled sole, 1.23~ 1.42 μm (1.32 ± 0.08 μm) and 1.15~1.20 μm (1.17 ± 0.02 μm) in brown sole, 1.22~1.44 μm (1.35 ± 0.09 μm) and 0.89~0.97 μm (0.92 ± 0.03 μm) in starry flounder, 1.25~1.67 μm (1.52 ± 0.16 μm) and 1.15~ 1.63 μm (1.40 ± 0.17 μm) in olive flounder, respectively. The chromatins of spermazoa of marbled sole, brown sole and starry flounder were compact and homogeneous, whereas that of olive flounder became granular and formed nonhomogeneous clumps. The nucleus of spermatozoa in each species had invagination, where the proximal centriole and distal centriole were existed. The proximal centriole was perpendicular to the distal centriole which was

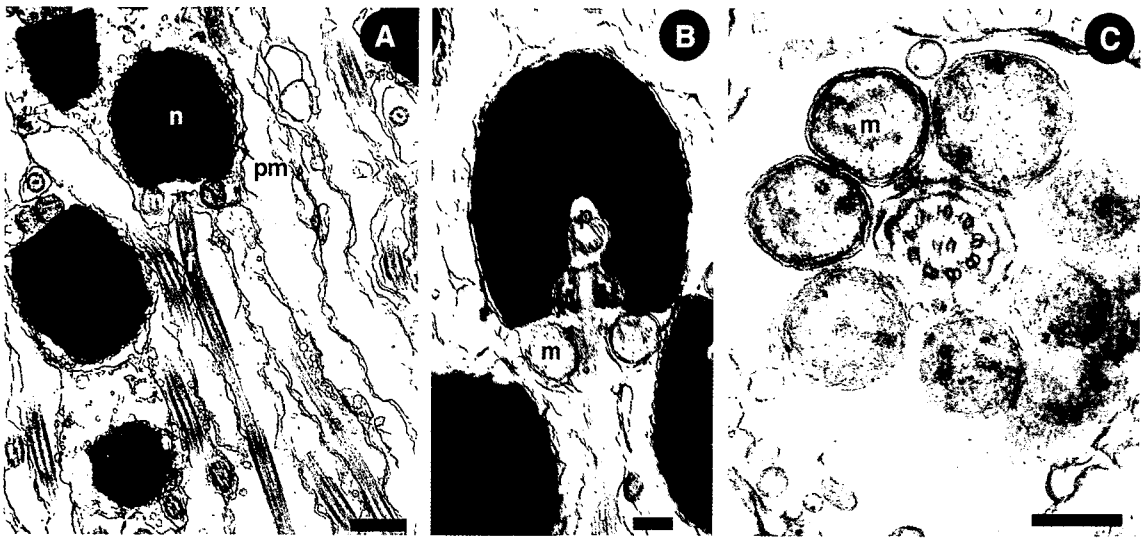

Fig. 2. Photographs of fresh spermatozoon of brown sole by transmission electron micro-scope. A: Sagittal section of a spermatozoon. Note the head with the compact chromatin and centriole. Bar=0.5 μm. B: Sagittal section of the head having proximal and distal centrioles. Bar=0.2 μm. C: Cross section of midpiece with seven mitochondria and 9+2 structure of flagellum. Bar=0.2 μm. d: distal centriole, f: flagellum, m: mitochondrion, n: nucleus, p: proximal centriole, pm: plasma membrane.

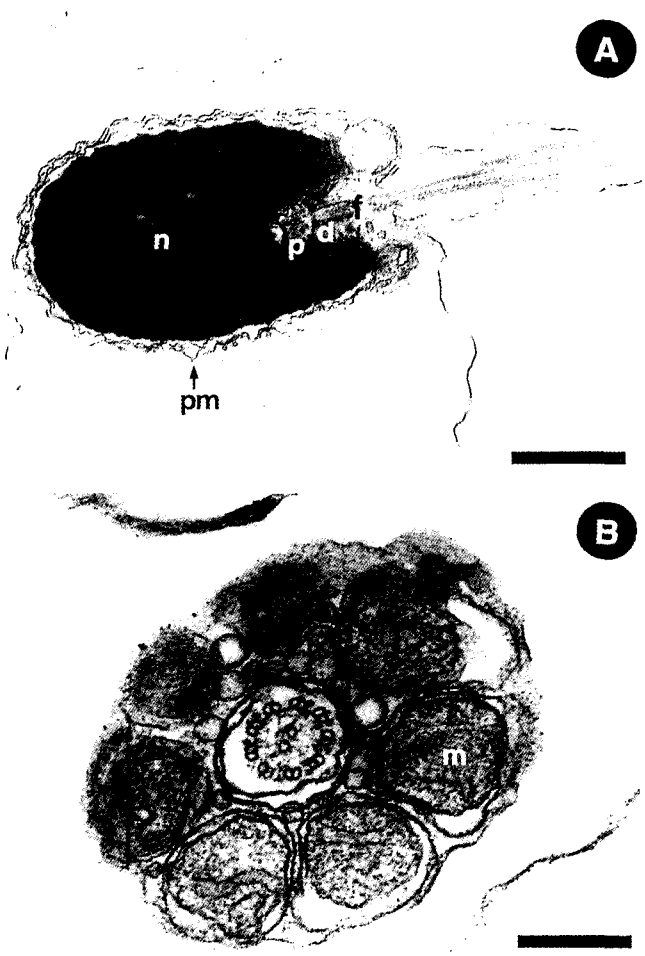

Fig. 3. Photographs of fresh spermatozoon of starry flounder by transmission electron microscope. A: Sagittal section of a spermatozoon. Note the head with the compact chromatin and centriole. Bar= 0.5 μm. B: Cross section of midpiece with seven mitochondria and 9+2 structure of flagellum. Bar=0.2 μm. d: distal centriole, f: flagellum, m: mitochondrion, n: nucleus, p: proximal centriole, pm: plasma membrane.

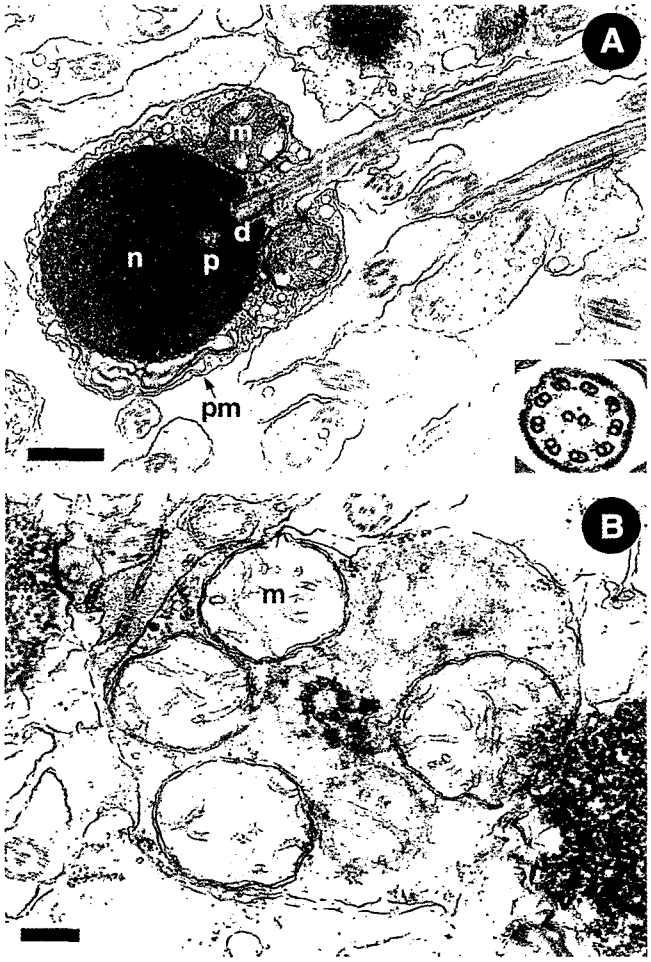

Fig. 4. Photographs of fresh spermatozoon of olive flounder by transmission electron microscope. A: Sagittal section of a spermatozoon. Note the head with the compact chromatin and centriole and the 9+2 structure of flagellum (insert). Bar=0.5 μm. B: Cross section of midpiece with six mitochondria. Bar=0.2 μm. d: distal centriole, f: flagellum, m: mitochondrion, n: nucleus, p: proximal centriole, pm: plasma membrane.

connected with flagellum (Figs. 1A, 1B, 2B, 3A and 4A). The numbers of independent mitochondria encircled the flagellum in the midpiece were eight in marbled sole, seven in brown sole and starry flounder and six in olive flounder. The flagellum had the typical 9+2 axoneme structure in four species (Figs. 1C, 2C, 3B, 4A and 4B).

Morphological changes of frozen spermatozoa

When the milts of four species of flatfishes were frozen with several appropriate conditions, almost no visible ultrastructural changes of freeze-thawed spermatozoa were observed, compared with fresh sperm. In a few cases, however, morphological alterations were observed (Figs. 5, 6, 7 and 8). The chromatin of spermatozoa of marbled sole was less condensed and homogeneous than that of intact spermatozoa (Fig. 5A). The plasma membrane was separated from the sperm head and the mitochondrial membrane in the midpiece was broken (Fig. 5B). In some cases, although the plasma membrane of the head part in spermatozoa of brown sole was broken and became wrinkled, no effect of the freeze-thawing process on the nucleus was evident. Its

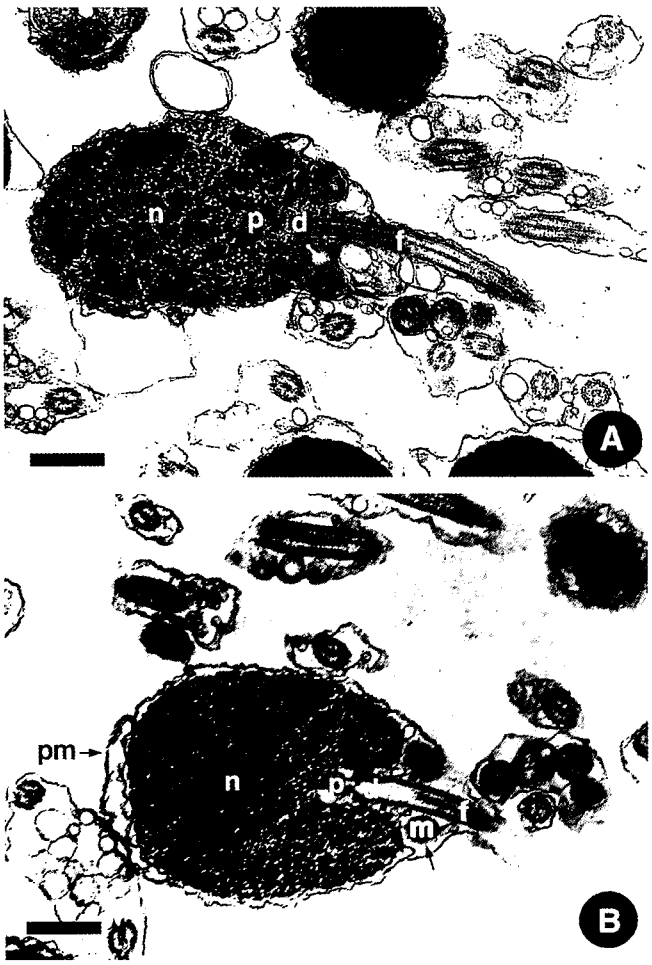

Fig. 5. Fig. 5. Photographs of post-thaw spermatozoon of marbled sole by transmission electron microscope. A: Sagittal section of a spermatozoon. B: Sagittal section of head part. Note the destroyed plasma membrane of head and mitochondrion (arrow). d: distal centriole, f: flagellum, m: mitochondrion, n: nucleus, p: proximal centriole, pm: plasma membrane. Bar=0.5 μm.

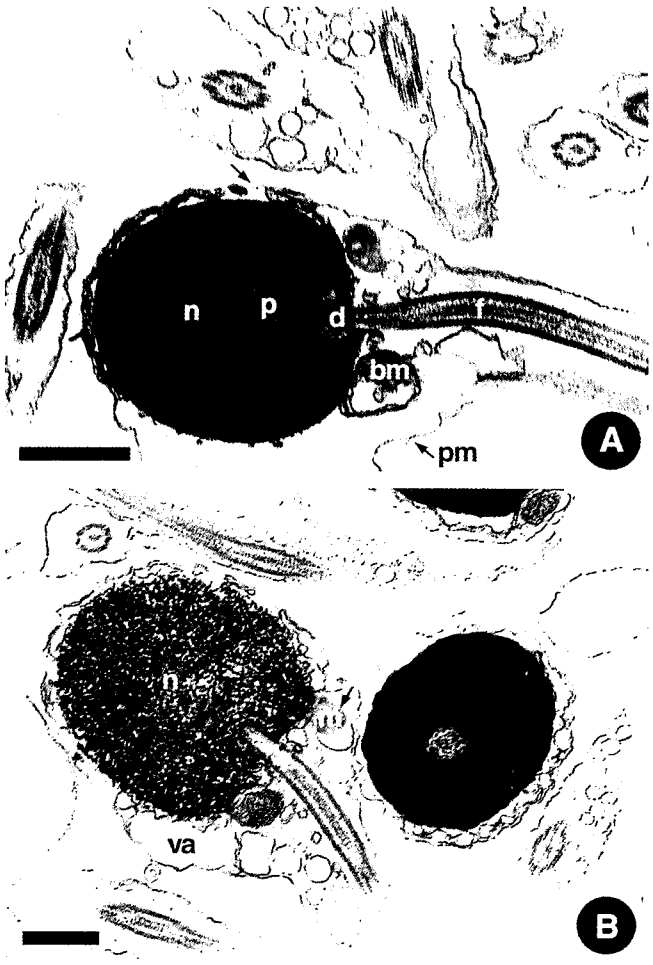

Fig. 6. Photographs of post-thaw spermatozoon of brown sole by transmission electron microscope. A: Sagittal section of a spermatozoon. Note the ruptured plasma membrane of head. B: Sagittal section of head part. Note formation of cytoplasmic vacuole. An arrow indicates the destroyed membrane of mitochondrion. bm: broken mitochondrial crista, d: distal centriole, f: flagellum, m: mitochondrion, n: nucleus, p: proximal centriole, pm: plasma membrane, va: vacuole. Bar=0.5 μm.

mitochondrial crista was destroyed (Fig. 6A). The chromatin was scattered and the plasma membrane exhibited vacuolization (Fig. 6B). In starry flounder, the plasma membranes of head and flagellum were excessively swollen or broken (Fig. 7A). The granulated chromatin was observed and the mitochondrial membrane was also destroyed (Fig. 7B). The plasma membrane of the head of olive flounder became loose (Fig. 8A). The chromatin consisted of many clumps of dense granules was scattered. In addition, extensive vacuolization was observed in the plasma membrane of the head and mitochondrial crista was broken (Fig. 8B).

Discussion

Understanding the physical and chemical properties of sperm and seminal plasma is very important to study the sperm physiology and to make a artificial seminal plasma used in sperm preservation. However, the information concerning the milt yields of several fish species is scarce. Milts of marbled sole, brown sole and olive flounder were far more viscous, and the sperm concentration and spermatocrit were considerably higher, compared with those of freshwater fish reported by other investi-

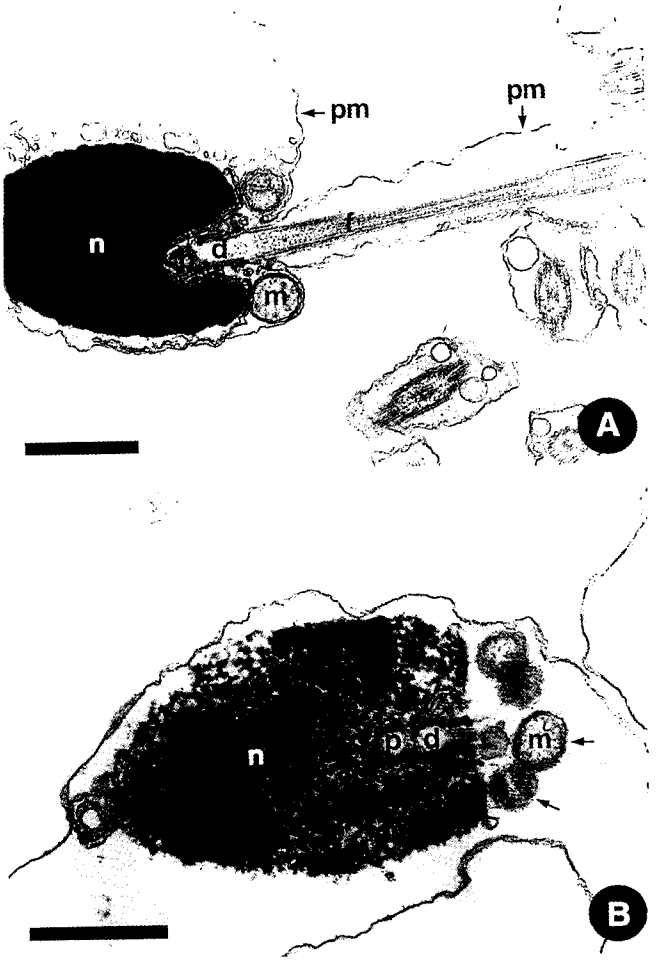

Fig. 7. Photographs of post-thaw spermatozoon of starry flounder by transmission electron microscope. A: Sagittal section of a spermatozoon. Note the pla-sma membrane swollen from head and flagellum. B: Sagittal section of head part. Arrows indicate the destroyed plasma membranes of mitochon-dria. d: distal centriole, f: flagellum, m: mito-chondrion, n: nucleus, p: proximal centriole, pm: plasma membrane. Bar=0.5 μm.

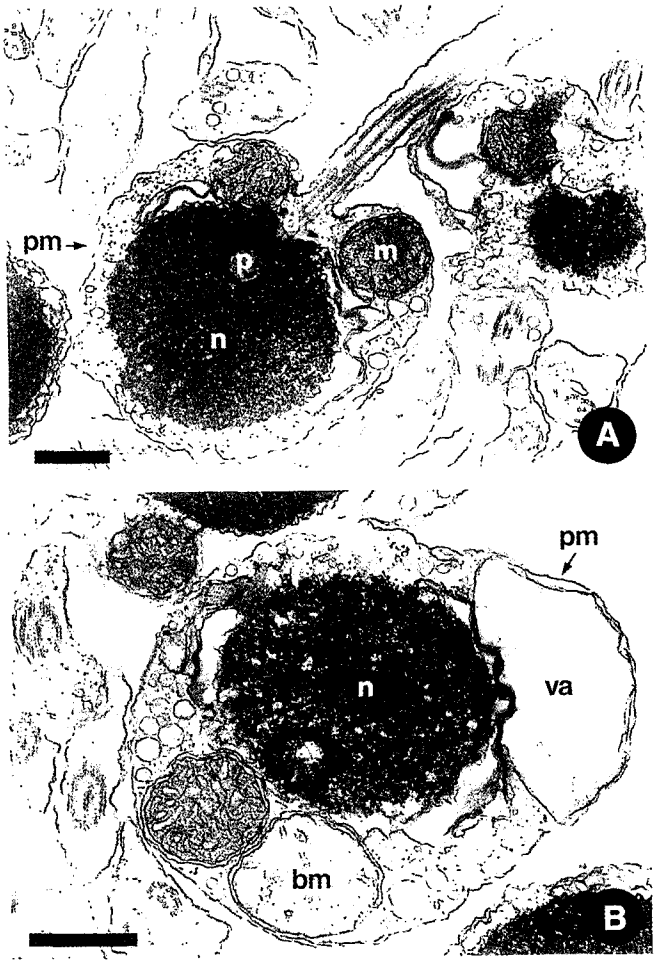

Fig. 8. Photographs of post-thaw spermatozoon of olive flounder by transmission electron microscope. A: Sagittal section of a spermatozoon. Note the plasma membrane swollen from head. B: Cross section of head part indicating the defected plasma membrane of head having a lot of cytoplasmic vacuoles. bm: broken mitochondrial crista, d; distal centriole, f: flagellum, m: mitochondrion, n: nucleus, p: proximal centriole, pm: plasma membrane, va: vacuole. Bar=0.5 μm.

gators (Chao et al., 1987; Ciereszko and Dabrowski, 1993; Lin et al., 1996; Piironen, 1985). The dense sperm concentration may be due to the hypertonicity of the seawater, causing possibly lower hydration in the testis during spermiation and leading to lower hydration and higher sperm concentration. Sperm concentration and spermatocrit in starry flounder were the lowest. These results may have been derived from the treatment of LHRH-a. Therefore, further studies should be performed to find out the effects of LHRH-a treatment on milt volume and sperm concentration.

Alkaline pHs offered the best conditions for maintaining the motility and fertility of sperm in marbled sole, brown sole, starry flounder and olive flounder. Wang and Crim (1997) reported that the pH of seminal plasma of ocean pout, *Macrozoarces americanus* tended to increase during the spawning season, corresponding with improved sperm motility. The sperm of halibut, *Hippoglossus hippoglossus* and sea bass, *Dicentrarchus labrax* exhibited the best motility at the pHs ranging from 7.5 to 8.5 (Billard et al., 1993) and pH 9 (Stoss, 1983), res-

pectively.

The osmolalities of seminal plasma in marbled sole, brown sole, starry flounder and olive flounder (328~337 mOsm/kg) were similar to those in black seabream, *Acanthopagrus schlegeli* (359 mOsm/kg in Morisawa, 1985; 382 mOsm/kg in Chang et al., 1995), puffer, *Takifugu niphobles* (342 mOsm/kg) (Morisawa, 1985), tiger puffer, *Takifugu rubripes* (383 mOsm/kg) (Chang et al., 1998), but higher than those in common carp, *Cyprinus carpio* (302 mOsm/kg), goldfish, *Carassius auratus* (317 mOsm/kg) (Morisawa et al., 1983b), rainbow trout, *Oncorhynchus mykiss* (297 mOsm/kg) (Morisawa, 1985) and river puffer, *Takifugu obscurus* (266 mOsm/kg) (Chang et al., 1999). Piironen (1985) suggested that lower osmolality of seminal plasma in freshwater teleosts might be partially caused by higher hydration in the testis. Especially, it seems that the lower osmolality in river puffer, although this species belongs to marine teleost, was derived from spawning habit in the river at spawning season.

Sodium, potassium, chloride and calcium are believed to exert their effects on the sperm by maintaining their osmotic balances. Variations in the concentrations of these elements and the ratios of sodium and potassium indicate a change of the cell membrane by an abnormal condition (Cruea, 1969). Also the ionic composition of seminal plasma has an important factor for sperm motility in fish. K^+ concentration in the seminal plasma of flatfish (4.0~20.8 mM/L; this study) was similar to those in black seabream (2.0 mM/L in Morisawa, 1985; 4.9 mM/L in Chang et al., 1995), puffer (5.3 mM/L; Gwo et al., 1993) and ocean pout (8.9 mM/L; Wang and Crim, 1997), but lower than those in muskellunge, *Esox masquinongy* (27.9 mM/L; Lin et al., 1996), rainbow trout (37 mM/L; Morisawa et al., 1983a) and comman carp (82.4 mM/L; Morisawa et al., 1983b). Quiescence of sperm motility in the seminal plasma of salmonids is associated with a high ratio of K^+ to Na^+ (Morisawa et al., 1983a). In marine teleosts, K^+ has no inhibitory effects on sperm motility in cod, *Gadus morhua* (Morisawa, 1985), flounder, *Platichtys flesus* and summer whiting, *Sillago ciliata* (Goodall et al., 1989), while K^+ enhanced the motility of carp sperm (Billard and Cosson, 1992).

Spermatozoon of olive flounder having a round head was similar to those of cyprinid fishes (Baccetti et al., 1984), tilapia, *Oreochromis* spp. (Bern and Avtalion, 1990), turbot, *Scophthalmus maximus* (Suquet et al., 1993) and muskellunge (Lin et al., 1996). Spermatozoa having ellipsoidal head in marbled sole, brown sole and starry flounder were similar to those in grayling, *Thymallus thymallus* (Lahnsteiner et al., 1992), rainbow trout (Lahnsteiner et al., 1996a) and puffer fishes (Miyaki et al., 1993). Independent mitochondria of midpiece in marbled sole, brown sole, starry flounder and olive flounder sperm encircled the flagellum. This structure exists also in other teleost species such as puffer (Gwo et al., 1993), Atlantic croaker, *Micropogonias undulatus* (Gwo and Arnold, 1992), rainbow trout (Lahnsteiner et al., 1996a) and *Mullus barbatus* (Lahnsteinier and Patzner, 1998). However, in common carp (Gwo et al., 1993) and muskellunge (Lin et al., 1996) the midpiece has an elongated sleeve with the scattered mitochondria. It is well known that the function of the mitochondria is to supply the energy required for movement and osmoregulation, normally provided by adenosine triphosphate, to the cell (Gwo and Arnold, 1992). Also the number of mitochondria determines the efficiency of energy supply (Lahnsteiner and Patzner, 1998). Further investigation is necessary to clarify the possible relation between abundant mitochondria and the duration of sperm movement in flatfish.

Most spermatozoa in marbled sole, brown sole, starry flounder and olive flounder were morphologically unaffected by cryopreservation. But in a few cases, the swelling and rupture of plasma membranes of the head, midpiece, and tail regions as well as those of the mitochondria were observed. Also the chromatin was granulated and the crista of the mitochondria was broken. The swollen and ruptured cell membranes indicate either a mechanical damage of the cells through intracellular ice-crystal growth or an instability of the membranes and their loss for osmoregulation (Lahnsteiner et al., 1996a). Gwo and Arnold (1992) suggested that the mechanism of damage during freezing and thawing was due to the lack of recognizable mitochondria, distortion of the 9+2 structure or the clumping of the flagella. This would render freeze-thawed spermatozoa nonmotile. Mechanical damage by freezing

and thawing of spermatozoa may be affected by the compositions of a diluent and a cryoprotectant, freezing rate and thawing rate. Therefore, it is necessary to investigate the effect of these conditions on morphological changes by studying the ultrastructures of spermatozoa during their freezing and thawing.

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