Activity of the Sperm of the Bluefin Tuna *Thunnus thynnus* under Fresh and Preserved Conditions

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Fresh semen of *Thunnus thynnus* from the Mediterranean Sea was very viscous and contained spermatozoa at high densities ($496-648 \times 10^6/\text{ml}$). When diluted with sea water, spermatozoa maintained high levels of motility for about seven minutes. From these characters of the semen, the dilution of semen with sea water or physiological saline seems to facilitate the operation of artificial insemination in this species. In experiments on the preservation of liquid semen in an ice bath, spermatozoa rapidly lost their motility and became completely inactive in four days, probably and partially due to the lowering of oxygen level in the highly viscous and dense semen. Cryopreserved semen in liquid nitrogen (cryoprotective agent: glycerol or DMSO; basic medium: Mounib's medium or 10% glucose) successfully maintained its spermatozoal motility, suggesting a high possibility of cryopreservation in the artificial insemination of *T. thynnus*. Glycerol showed its cryoprotective effectiveness at equilibration times longer than 20 minutes, while DMSO was effective at equilibration times shorter than 20 minutes.

Recent advances in the mass production of marine fish seedlings have depended largely on the success in rearing of spawners and the induction of their spontaneous spawning in captivity. Yet sperm preservation and artificial insemination are useful and often necessary techniques when farm-raised spawners are hardly available or when basic knowledge of reproductive biology is required for newly establishing a seedling production system of a species. This is particularly the case with *Thunnus* species in which their huge sizes restrict the opportunity of rearing through to maturity.

During our research of tuna spawning in the Mediterranean, we collected and examined the sperm of the bluefin tuna *Thunnus thynnus* LINNAEUS (Scombridae). This paper presents the results of our observations on the activity of the sperm of this species under fresh and preserved conditions, in order to provide data on the availability of sperm in artificial insemination.

Material and Methods

Collection of Semen

Semen was collected from six males, each measuring about 300 kg in body weight, caught by a set-net off Favignana Island near Sicily, Italy, from

29 May to 12 June 1980. Surface water temperature at the fishing ground ranged from 17.0° to 20.7°C. When these specimens were hauled on board, they released semen without being pressed on their abdomens. After removing released semen, uncontaminated semen was collected, using an injector, in open test tubes. These test tubes were placed in crushed ice in an ice-box, and transported to Favignana. Semen from different individuals was not mixed.

Measurement of Sperm Activity

The activity of sperm was examined in terms of the motility of spermatozoa and the mode of movement of motile spermatozoa. Motility was expressed by the following six-grade scores: score 5, more than 80% of spermatozoa motile; score 4, 60-80%; score 3, 40-60%; score 2, 20-40%; score 1, nearly 0-20%; score 0, few spermatozoa motile. The mode of spermatozoal movement was classified into forward movement (≡), circular movement (≡), and vibratory movement (+), following NOMURA.¹⁾

Activity of Fresh Sperm

Immediately after 40-50 minutes' transportation in a container with ice, the motility and mode of movement were observed every minute after dilution with sea water in a volume rate of about 1:100 at room temperatures (18.0°-20.2°C).

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Preservation of Liquid Semen

Five ml of fresh undiluted semen was placed in a plastic bowl and preserved in an ice bath kept covered with a wet towel, which was replaced when it became dry. Care was taken not to let water drop in the bowl from the towel. Spermatozoal activity was examined at semen-sea water dilution rates of 1: 3–5 (such low rates, compared with the rate used in the foregoing experiment with fresh semen, were employed merely for the sake of quick manipulation for checking spermatozoal activity for a short period).

Cryopreservation

Before freezing, semen was mixed with a diluent composed of seven parts of a basic medium and one part of a cryoprotective agent. Two different basic media were used, i.e., the Mounib's medium (125 mmol sucrose, 65 mmol reduced glutathione, and 100 mmol potassium bicarbonate)²⁰ and 10% glucose. The cryoprotective agents used were glycerol and dimethylsulfoxid (DMSO). The diluents were stored in an ice bath and used within 24 hours after preparation. Diluted semen was equilibrated in an ice bath for 10–30 minutes. Samples of about 0.8 ml of semen were frozen in 1-ml plastic straws, sealed with straw powder, in a liquid nitrogen freezer. Freezing was carried out in two steps; the straws were immersed in nitrogen vapor in the freezer for three minutes before placing them in liquid nitrogen. Samples were thawed by putting the straws in water at 40°C until the semen became liquefied and then transferring the straws into an ice bath.

Comparative Material

Semen of the frigate mackerels, *Auxis thazard*

and *A. rochei*, chum salmon, *Oncorhynchus keta*, and rainbow trout, *Salmo gairdneri*, all collected from Japanese waters, was examined for comparison of spermatozoal density with *Thunnus thynnus*.

Results

Fresh Semen

The semen of *Thunnus thynnus* was very viscous like a white oil paint. The density of spermatozoa (from four males) was 496–648 $\times 10^8$ /ml. These densities are considerably higher than those of the other four species presently examined, particularly the two species of the Salmonidae (Table 1).

Immediately after dilution with sea water, spermatozoa (from three males) were rather highly active, with a motility score of 4 (two individuals) or 3 (one individual) and with forward movement (++) in all samples (Fig. 1). The motility score dropped to 3.5 or 3 four minutes after dilution, and this level of motility was maintained until

Table 1. Comparison of the density of spermatozoa in the semen of *Thunnus thynnus*, *Auxis thazard*, *A. rochei*, *Oncorhynchus keta* and *Salmo gairdneri*

Species	Density ($\times 10^8$ /ml)		No. of specimens examined
	Range	Average $\pm$ SD	
<i>Thunnus thynnus</i>	496~648	594 $\pm$ 72	4
<i>Auxis thazard</i>	296~567	442 $\pm$ 102	5
<i>A. rochei</i>	223~500	386 $\pm$ 136	4
<i>Oncorhynchus keta</i>	216~256	241 $\pm$ 22	3
<i>Salmo gairdneri</i>	126~218	177 $\pm$ 39	4

Fig. 1. Change in the activity of fresh sperm from three individuals of *Thunnus thynnus* after dilution with sea water. Where different modes of spermatozoal movement (+, ++) were observed at the same time, mode observed more frequently was placed above that observed less frequently.

Fig. 2. Change in the activity of sperm from two individuals of *Thunnus thynnus* in liquid-state preservation with water-ice. The presentation of spermatozoal movement by markings is the same as in Fig. 1.

seven minutes after dilution. However, circular movement (++) predominated over forward movement (++) seven minutes after dilution. Then the motility declined gradually and reached score 0 around 14 minutes after dilution (Fig. 1). Even when the motility score reached 0, surviving spermatozoa were observed performing slow forward movement or circular movement, and little vibratory movement (+) was noticed. Spermatozoa were completely immotile 20–27 minutes after dilution.

Preservation of Liquid Semen

Spermatozoa (from two males) immediately before preservation showed a motility score of 5.

This score, higher than in the foregoing experiment, may have resulted from the difference in dilution rate with sea water (1:3–5 in this case and about 1:100 in the foregoing experiment). The score dropped to 2.5 (one individual) or 2 (the other individual) about 24 hours after the start of preservation (Fig. 2). Spermatozoal movement gradually shifted from ++ to +. Then the motility continued to decrease, and the score reached 0 and spermatozoa became totally immotile in four days. As was in the foregoing experiment with fresh semen, we observed forward and circular movements, but not any clear vibratory movement, until spermatozoa became totally immotile. The semen appeared more viscous in liquid-state preservation than in fresh semen.

Cryopreservation

Table 2 shows motility scores of frozen-thawed semen preserved at a constant semen-diluent rate of 1:3 and with different combinations of basic medium, cryoprotective agent, and equilibration time. Between the two cryoprotective agents, i.e. glycerol and DMSO, contrary tendencies were noticed in the relationship of equilibration time and spermatozoal motility. Namely, the motility score tended to be higher at longer equilibration times when glycerol was used, while the score was higher at shorter equilibration times with DMSO. As to the effects of the two basic media, i.e. the Mounib's medium and 10% glucose, no significant difference was recognized when glycerol was used as a cryoprotective agent. Within a preservation period of 23 days, the motility of spermatozoa after thawing did not decline in all preserving conditions prepared.

Table 2. Spermatozoal motility scores of cryopreserved-thawed semen of *Thunnus thynnus* at various combinations of cryoprotective agent, basic medium and preservation period. Figures in parentheses are motility scores when sea water was added to thawed semen at a ratio of about 1:2

Cryoprotective agent	Basic medium	Equilibration time (min)	Preservation period (days)		
			3	8	23
Glycerol	Mounib's medium	10	2.5 (1.5)	1 (2)	2 (2.5)
		20	2 (1)	2 (1)	1 (1)
		30	3 (3)	4 (3)	3 (3.5)
	10% Glucose	10	1 (1)	1 (0.5)	0.5 (0.5)
		20	0.5 (1)	3 (3)	3 (2.5)
		30	3 (2.5)	3 (3)	3 (2.5)
DMSO	Mounib's medium	10	5 (3)	4 (3)	
		20	3 (1.5)	3 (1)	3 (2)
		30	2.5 (2)	3 (2)	3 (2)

Discussion

The nature of sperm of *Thunnus thynnus* differs considerably from that of salmonid sperm. Compared with salmonids, the semen of *T. thynnus* is far more viscous, the density of spermatozoa is considerably higher (Table 1), and spermatozoal activity (Fig. 1) is maintained for a much longer period when diluted with water (for spermatozoal survival of salmonids, see GINZBURG³⁾). From these data it follows that the dilution of semen with sea water or physiological saline seems to facilitate the operation of artificial insemination of this species without affecting the fertilizing ability. The difference in initial motility at different seawater dilution rates of semen examined at fresh state (Figs. 1, 2) suggests that low semen-water dilution rates, 1:3 for example, bring higher motility and consequently higher fertility.

The rapid decrease in spermatozoal motility in liquid-state preservation (Fig. 2) is quite in contrast to the long survival of spermatozoa under similar conditions found in salmonids⁴⁻⁸⁾ and some marine species such as *Clupea harengus*⁷⁾ and *Mugil capito*.⁸⁾ Though the dilution of semen rendered less favorable results in an attempt at liquid-state preservation of rainbow trout sperm,⁹⁾ the high viscosity of semen and high density of spermatozoa in *Thunnus thynnus* may cause a quick lowering of oxygen level in the semen, and this may be a partial cause of the shortening of spermatozoal survival. BÜYÜKHATİPOĞLU and HOLTS⁶⁾ showed that, in their attempt to preserve trout sperm at 4°C under various gas conditions, the highest survival was seen in samples preserved under oxygen gas. Some modifications, possibly such as the creation of aerobic conditions, are necessary to achieve a better survival of spermatozoa in the preservation of liquid semen of *T. thynnus*.

Although fertilizing ability could not be examined in this study due to our failure to obtain ripe eggs, the successful maintenance of spermatozoal motility in cryopreserved-thawed semen indicates a high possibility of utilizing this technique

in the artificial insemination of *T. thynnus* as in some other marine fishes.^{2, 9-11)} The results of this study (Table 2) show that both glycerol and DMSO can be effective cryoprotective agents, provided that the equilibration time is longer than 20 minutes in the former and shorter than 20 minutes in the latter.

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