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Preliminary experiences for cryopreservation of *Sparus aurata* and *Diplodus puntazzo* semen

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SUMMARY - The feasibility of cryopreserving gilthead seabream, *Sparus aurata*, and sharpsnout seabream, *Diplodus puntazzo,* semen with simple and reliable techniques was investigated. The preliminary results of these trials, which were conducted over two seasons, are reported here. In trial 1, during the first season, semen of gilthead seabream was cryopreserved in cryovials using a programmable cooler. In trial 2, in the second season, gilthead semen was cryopreserved in 0.5 ml straws and cooled on a rack at a fixed distance from LN_2. In trial 2 it was possible to conduct fertility trials. Results were positive in trial 1 with high motility scores of sperm after cryopreservation with DMSO and NaCl 1% in the proportion 10:15:75 v/v. In trial 2 the fertilizing ability of cryopreserved semen was demonstrated with significantly ($P<0.05$) different results in relation to gametes contact time of 5 and 10 min. For sharpsnout seabream, the methods employed were similar to those used in trial 1 for gilthead seabream, but the results were poorer. The spermatozoa that survived to cryopreservation had low motility scores. These results are to be considered a starting point for reaching a reliable cryopreservation protocol for the semen of this species.

Key-words: *Diplodus puntazzo, Sparus aurata*, cryopreservation, semen, spermatozoa, fertilization.

RESUME - *"Expériences préliminaires pour la cryoconservation de sperme de* Sparus aurata *et de* Diplodus puntazzo*." Cet article étudie la faisabilité de la cryoconservation du sperme de daurade royale,* Sparus aurata *et de sar à museau pointu,* Diplodus puntazzo *avec des techniques simples et fiables. Les résultats préliminaires de ces essais, qui ont été menés sur deux saisons, sont reportés ici. Dans le premier essai, pendant la première saison, le sperme de daurade royale a été cryoconservé dans des cryoampoules en utilisant un refroidisseur programmable. Dans le second essai, pendant la deuxième saison, le sperme de daurade royale a été cryoconservé dans des paillettes de 0,5 ml et réfrigéré sur un portoir à une distance fixe de l'azote liquide. Dans l'essai 2, il a été possible de mener des tests de fertilité. Les résultats ont été positifs pour l'essai 1 avec de forts résultats de motilité du sperme après cryoconservation avec du DMSO et NaCl 1% selon une proportion 10 : 15 : 75 v/v. Dans l'essai 2, l'aptitude à la fertilisation du sperme cryoconservé a été démontrée avec des résultats significativement différents ($P<0,05$) selon le temps de contact des gamètes, de 5 et 10 minutes. Pour le sar à museau pointu, les méthodes employées ont été semblables à celles utilisées dans l'essai 1 pour la daurade royale, mais les résultats ont été moins bons. Les spermatozoïdes ayant survécu à la cryoconservation avaient de faibles performances de motilité. Ces résultats sont à considérer comme un point de départ pour mettre au point un protocole fiable de cryoconservation pour le sperme de cette espèce.*

Mots-clés : Diplodus puntazzo, Sparus aurata, *cryoconservation, sperme, spermatozoïdes, fertilisation.*

Introduction

Cryopreservation of spermatozoa is an important procedure for facilitating controlled reproduction of fish, allowing hybridization of species with non-overlapping

reproduction period, self-fertilization of protandrous fishes such as the gilthead and sharpsnout seabream, conservation of endangered species, (Gwo *et al.* 1991) facilitating the shipment of genetic material and the genetic selection of beneficial traits (Chambeyron and Zohar, 1990).

In recent years the application of low temperature preservation techniques has been applied to an increasing number of new aquatic species of commercial and conservational interest, mostly belonging to three groups: the Salmonoids, tilapia and carp (Rana, 1995).

In the Mediterranean, the Sparid gilthead seabream, *Sparus aurata*, has become one of the most important cultured marine species and sharpsnout seabream, *Diplodus puntazzo*, which is considered one of the fastest growing Sparid, is becoming an important reared species; Sparid aquaculture is gaining importance in aquaculture (Barbato and Corbari, 1995).

The aim of the present study was to conduct preliminary trials to develop a simple and reliable technique for cryopreserving gilthead and sharpsnout seabream semen. Previous studies concerning this topic on *Sparus aurata* were reported by Billard (1984), with successful fertility trials with cryopreserved semen and by Chambeyron and Zohar (1990) on an efficient diluent for semen cryopreservation. Similar information on cryopreservation of *Diplodus puntazzo* semen, however, is still unavailable. The present study was based on earlier trials at ENEA by Villani and Catena (1991, 1992) with seabass, *Dicentrarchus labrax,* spermatozoa, on methods used by Gwo *et al.* (1991) on the Sciaenid Atlantic croacker, *Micropogonias undulatus*, spermatozoa and on the general paper by Rana (1995) on the cryopreservation of fish spermatozoa.

Materials and methods

Procurement of gametes

Mature sharpsnout and gilthead seabream broodstocks were obtained from commercial farms in Puglia and Sardinia, Italy, respectively, maintained in 2.6m and 2m diameter recirculating tanks at the Casaccia ENEA centre, near Rome, Italy, and fed on a commercial seabream diet. Gilthead seabream broodstock were reared in the ENEA facility for one year, whilst sharpsnout were conditioned in the recirculating system for just two weeks before the first milt sample was taken. To collect milt, fish were anaesthetized and, after their weight and length were measured, gentle pressure was exerted on the abdomen to expel urine. Their abdomen was dried with a soft cloth and then each fish was manually stripped for milt. The expressed milt was collected in 15 ml polypropylene tubes or in Petri dishes and held on crushed ice. Aliquots were used for microscopic examination to check motility, after a ten fold dilution with sea water, and to evaluate the spermatozoa concentration, after a dilution $1:10^3$ with 1%NaCl in distilled water. Sperm motility was scored on a subjective scale between zero and five, zero being no motility and five, maximum (80-100%) motility (Chambeyron and Zohar, 1990). Spermatozoa concentration was estimated by Thoma haemocytometer counts.

Oocytes were obtained through catheterization from a single three year old gilthead seabream female, ripened at the ENEA facility.

For gilthead seabream, two separate trials were conducted: the first after the fish arrived to the ENEA facility and the second, one year later.

Trial 1

Gilthead and sharpsnout seabream milt was diluted ten fold with 1% NaCl containing a final concentration of 15% dimethyl sulfoxide (DMSO) (Gwo *et al.*, 1991).

1 ml aliquots of diluted milt were dispensed in 1.5ml cryovials and cooled in a programmable freezer at -10°C/min to -50°C, then rapidly cooled (-50°C/min) to -130°C followed by immersion in liquid nitrogen. All frozen samples were stored in a cryocontainer and thawing was executed in a warm bath at 26°C for 2 minutes. Post-thawed motility was immediately assessed under the microscope after dilution in sea water.

Trial 2

Fresh milt from individual gilthead seabream males were checked for quality and pooled; only milt with a motility score of five was used. Pooled milt was diluted as described above and stored in 0.5ml straws, then cooled by placing straws horizontally, 4.3 cm above LN_2 in a covered polystyrene box. After cooling for 10min straws were plunged and stored under LN_2.

To test sperm viability, lots of oocytes (165±6, S.E.) were divided into two groups: group A, with 4 lots examined once at the end of the incubation and group B with 6 lots examined two or three times during the incubation period. Oocytes were fertilized with post-thawed spermatozoa at a rate of 260,000±9,000 spermatozoa/oocyte, in 2.4 ml of seawater. For 2 lots of group A and 3 lots of group B the contact time between the gametes was set for 5min, whilst in the remaining groups it was set for 10 min. The 10 lots of fertilized oocytes were incubated in beakers containing 500 ml of seawater and maintained at the constant temperature of 17.5°C with gentle aeration.

Results

Trial 1

The first trial with gilthead seabream was carried out on 15 males and sperm production was effective for at least 30 days under the captivity conditions at the ENEA centre. Fish size varied and ranged from 118 to 444 g in weight and from 22.0 up to 31.5 cm in total length. Milt volume ranged from 0 to 3.1 ml. Only 3 out of 15 fishes did not yield any milt.

The spermatozoa concentration in the freshly collected milt samples ranged from $11.6*10^9$ to $26.7*10^9$ spermatozoa/ml with an average of $17.95*10^9$. The relations between fish weight and milt volume and sperm concentration are shown in Fig. 1.

Freshly collected spermatozoa showed motility values of four and five when diluted in 1:10 sea water.

One week after freezing, post-thaw motility values of three and four were observed in samples, derived from 4 different fish.

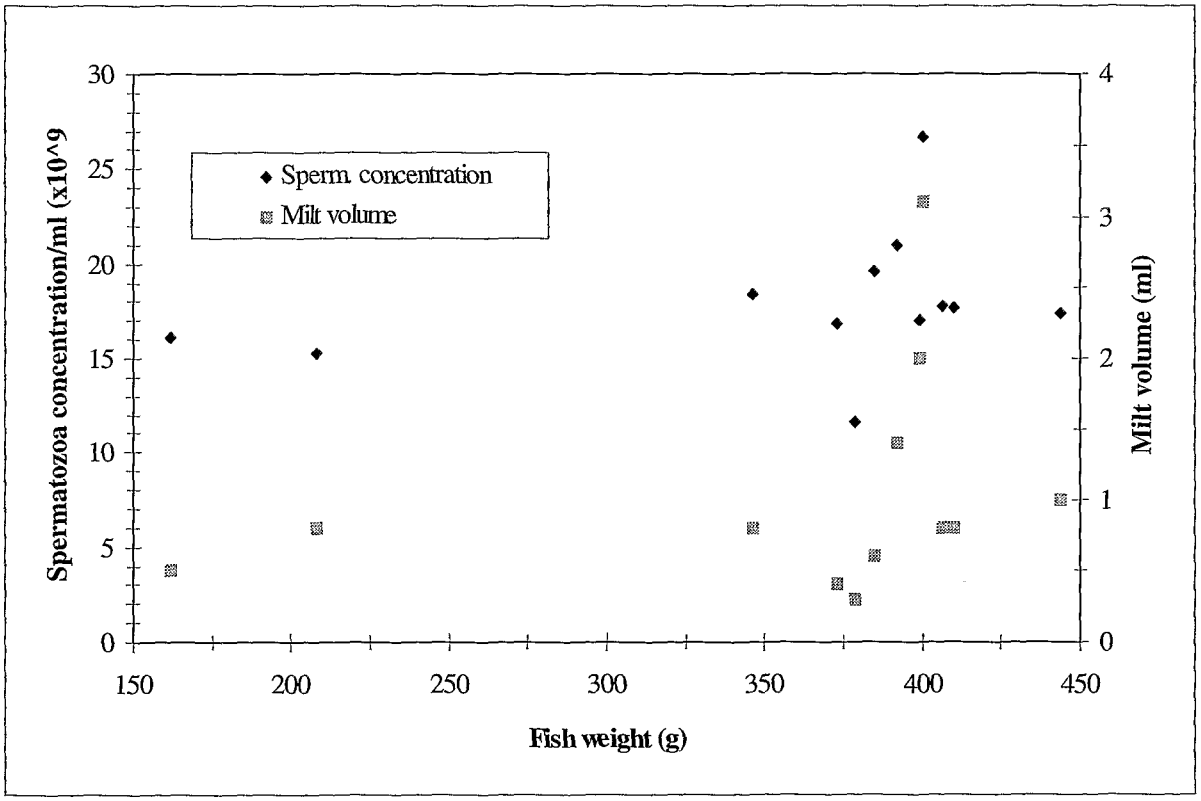

Fig. 1. Relations between fish weight, milt volume and spermatozoa concentration per ml of milt (Trial 1).

Out of 16 sharpsnout seabream males, only two produced milt of useable quality and quantity. The motility of fresh milt after activation with filtered sea water was rated as three. Samples checked after thawing and activation gave a score of two for one fish, one for the other fish sampled.

Trial 2

An average of 300µl of milt was obtained from four gilthead seabream males, and 4 g of oocytes was collected from one female through catheterization.

The sperm density of pooled gilthead seabream milt was $14.15*10^9$/ml and post-thaw motility after activation with sea water was estimated to be four. The effect of contact time and handling connected to the embryonic development checks on hatch rate are shown in Table 1 with data concerning the different lots for fertility trials. Increasing the contact time from 5 to 10 min significantly ($P<0.05$) decreased the hatch rate. After 61h of incubation 27.4 and 22.5% of the group B eggs, having a contact time of 5 and 10 min, respectively, developed into embryos and hatched larvae. For group A, after 66 h of incubation, corresponding rates were 51.1 and 19.3%. The effect of handling during embryonic development was only significant at $P< 0.075$.

Table 1. Data on fertility trials with gilthead seabream frozen semen (Trial 2)

Contact time between gametes(')	Group	No. of intermediate handlings	Initial No. of eggs	No. of embryos+ hatched larvae	% of embryos+ hatched larvae
5	A	0	145	85	58.6
5	A	0	160	71	44.4
5	B	1	194	52	26.8
5	B	1	143	24	16.8
5	B	2	160	60	37.5
10	A	0	194	33	17.0
10	A	0	159	35	22.0
10	B	1	148	50	33.8
10	B	1	166	37	22.3
10	B	2	184	25	13.6

Discussion

The results obtained in the present study for gilthead seabream show promise for developing a reliable and simple method of cryopreserving the semen of this species.

Better results were obtained in Trial 2, employing the simple polystyrene box for freezing, 0.5 ml straws and pooled sperm. These better results may be due to the narrower space between the sperm and the LN_2; the thin walled straws facilitate rapid heat transfer (Rana, 1995), compared with cryovials. As for pooled sperm, there are different evaluations on the advantages of mixing sperm from different

males together, taking into account the fertilization rate as a test. Legendre and Billard (1980) reported positive results for rainbow trout whilst Stoss and Holtz (1983) reported results with no distinct differences between pooled and non-pooled, working on the same species.

Data concerning milt volume and spermatozoa count in relation to fish weight (Trial 1) are reported in Fig.1 to show the variability and the irregular trends for these relations, indicating the presence of other factors, different from weight, influencing the semen production. Correlation tests between weight and both milt volume and spermatozoa counts were not significant.

The best extender and cryoprotectant tried by Gwo *et al.* (1991), that is NaCl 1% and DMSO respectively, for the Atlantic croaker, and applied in the present study, seem to work rather well also with gilthead seabream, in addition to several other species as indicated in their paper.

There was a significant ($P<0.05$) difference between the 5 min and 10 min gamete contact time on the final development rate. The cryoprotectant DMSO, may in fact have a cytotoxic effect, so it may be important to find the most convenient contact time for a given DMSO concentration to avoid possible toxic effects on the gametes and to maximise fertilization rates. The handling of eggs during incubation could have reduced the final development rate.

The preliminary procedure described here for cryopreserving sharpsnout seabream sperm was not as successful as with gilthead seabream. One of the possible cause for the poorer result is that the sharpsnout arrived at the ENEA centre towards the end of the spawning season, when the sperm quality is usually poor, as reported by Billard (1984) for seabass. Rather bad conditions, due to handling and travel stress. Future trials will hopefully define more precisely reliable cryopreservation protocols.

Sparid aquaculture is becoming increasingly important, and only a few species have been tested for semen cryopreservation. Notably, there is one report on a Japanese species, *Evynnis japonica* or crimson seabream (Kurokura *et al.*, 1986) and the works already mentioned on *Sparus aurata.* It is therefore advisable the increase of studies on the cryopreservation of gametes from Sparid species in the near future, in view of the connected benefits already mentioned in the introduction.

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