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Cryopreservation of the spermatozoa of *Crassostrea tulipa* and three other oysters

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ABSTRACT

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The application of a simple recipe of a cryoprotective diluent to the spermotozoa of four species of oysters is reported. Different concentrations of DMSO in a diluent containing 0.6% glycine (*v*/*v*) in seawater were used to store the spermatozoa in a liquid nitrogen freezer (−190°) for periods ranging from 12 to 217 days depending on the availability of eggs for test fertilization. There was an evidence of interspecific differences in the retention of viability of the cryopreserved spermatozoa. The highest mean fertility rate (93%) was achieved with the frozen spermatozoa of *C. gigas* in 10% DMSO, followed by *S. cucullata* and *C. tulipa* with 78.3% and 71.4% mean fertility respectively, both in 15% DMSO. The highest mean rates of larval yield were 54.7% in 20% DMSO for *C. tulipa*; 51.0% in 15% DMSO for *S. cucullata*; and 17.8% in 20% DMSO for *C. gigas*. For *C. iredalei* the optimal DMSO concentration for retention of sperm motility was 15%.

The addition of glycine to the cryoprotective diluent enhanced the overall viability of the spermatozoa of *C. tulipa* (the only species tested) but did not influence larval vigour. The larvae from cryopreserved spermatozoa of this oyster, reared for 15 days, did not exhibit latent freeze-induced injury.

INTRODUCTION

The cryopreservation of the spermatozoa of oysters has been a subject for investigation (see Lannan, 1971; Hughes, 1973; Staeger, 1974; Zell et al., 1979; Van der Horst et al., 1985; Bougrier and Rabenomanana, 1986) primarily because of the possibility of their utilization for self-fertilization in these protandric hermaphrodites. This would increase the rate of inbreeding for valuable traits such as rapid growth, disease resistance, better shape, texture and flavour. A success in the cryopreservation of the sex products would also ensure ready supply of gametes for year-round hatchery seed production with-

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out the costly conditioning of adults. It could also provide an easy means of shipment to different locations.

All the earlier workers cited above employed dimethyl sulphoxide (DMSO) as the cryoprotective agent, but in different experimental protocols and hence achieved varying degrees of success ranging from zero to 91% fertilization. This necessitates the determination of optimal concentrations of this agent whenever new recipes and/or new species of oysters are being investigated.

So far only two species of oysters (*C. gigas* (Thunberg) and *C. virginica* (Gmelin)) have been the subjects for cryobiological investigation of bivalve gametes. Also there has been only one brief documentation of growth of oyster larvae obtained from eggs fertilized with cryopreserved spermatozoa (Zell et al., 1979). This paper reports on the application of a simple cryopreservation recipe to four economically important species of oysters originating from three different geographical areas, namely West Africa, Indonesia and the United Kingdom.

MATERIALS AND METHODS

Source of gametes

Gametes for the work were obtained from four different species of oysters, namely *Crassostrea tulipa* (Lamarck) from Ghana (West Africa), *C. iredalei* (Faustino) and *Saccostrea cucullata* (Born) both from Indonesia and *C. gigas* obtained locally, but having originally been introduced from British Columbia between 1964 and 1972 (Walne, 1979). The supply of viable gametes was limited by the ability of the oysters to remain in good reproductive state under laboratory conditions.

All gametes used in the experiments were extracted from the gonadal tissue using a Pasteur pipette after removing the top valve of the oyster shell.

Diluent and freeze-thaw protocol

The cryoprotective solution used as the diluent consisted of concentrations of DMSO (*v*/*v*) ranging from 5 to 20% at 5% intervals prepared with filtered (0.2 μm), UV-irradiated seawater (32‰) containing 0.6% glycine. In all experiments approximately 0.2 ml of spermatic extract was mixed with 1.5 ml of the diluent in sterile polypropylene cryotubes of 1.8 ml capacity. Active spermatozoa, of motility rating of 4 or 5 (based on the scale of Legendre and Billard, 1980), were used for the storage experiments. Cryotubes containing the spermatozoa suspensions (SS) were held on ice and conveyed to the freezing facility within 30 min of extraction from the gonad. The SS were cooled at the rate of 4.7°C min^{-1} to −70°C and transferred into a liquid nitrogen freezer (Linde Model Super 30A) for storage at −190°C. Frozen SS were subjected to quick thawing by immersing the cryotubes in a water bath at 55°C for 20 s.

The effect of different concentrations of DMSO on the viability of the cold-stored spermatozoa was tested in all the four species of oysters. Later, the influence of glycine as an additive was tested using spermatozoa from *C. tulipa*. In this experiment diluents without 0.6% glycine served as 'controls' for comparison.

Evaluation of the viability of cold-stored spermatozoa

Fertilizations were conducted by mixing 0.5 ml of thawed SS with 2000 eggs (fresh) held in 200 ml of 'treated' seawater in duplicate vessels (250 ml tall form pyrex glass beakers). The cultures were decanted 3 h later and fresh treated seawater was added. Incubation temperatures were 30°C for the tropical oysters and 25°C for *C. gigas*. The viability of the spermatozoa was evaluated 24 h after fertilization by examining subsamples in a Sedgewick Rafter cell. Percentage fertilization (%F) was calculated by pooling abnormal embryos and normal D-larvae, while larval yield (%D) was based on the proportion of the D-larvae in a sample.

Growth, survival and competence (formation of eyespots) of *C. tulipa* larvae obtained from cryopreserved spermatozoa were compared with those from fresh sperm. One-day-old larvae were reared at 27°C and fed with a mixed diet of *T.* Iso (see Ewart and Epifanio, 1981) and *Monochrysis lutheri*. The culture method was similar to that described by Yankson and Moyse (1983). After 15 days, each culture was subsampled and the first 30 larvae (well positioned) were measured for shell height and length using a calibrated ocular micrometer. The numbers of competent larvae and dead veligers were recorded.

For *Crassostrea iredalei* no reproductive adults were available to provide eggs for test fertilization. Instead, motility (%M) of the cold-stored spermatozoa was estimated by counting motile and non-motile individuals using a haemocytometer after approximately 30× dilution of the thawed SS.

RESULTS

Effect of DMSO concentration

The effect of concentration of DMSO in the diluent on the viability of the cold-stored spermatozoa of three species of oysters is presented in Fig. 1. The one-way classification analysis of variance using $\log X$ or $\log (X+1)$ transformation wherever appropriate (see Steel and Torrie, 1960), showed that the influence of concentration of DMSO on both %F and %D was significant at $P<0.005$ for *C. tulipa* and *C. gigas*, and at $P<0.01$ for *S. cucullata*. By inspection, the optimal DMSO concentration for %F was in the range 15–20% for *C. tulipa*, producing 70.1–71.4% mean fertilization rates. *C. gigas* had an optimal concentration range of 10–20% DMSO with mean fertilization rates of 89.7–93.0%. The optimal concentration for *S. cucullata* was 15% giving a mean fertilization rate of 78.3%. The optimal DMSO concentrations for %D

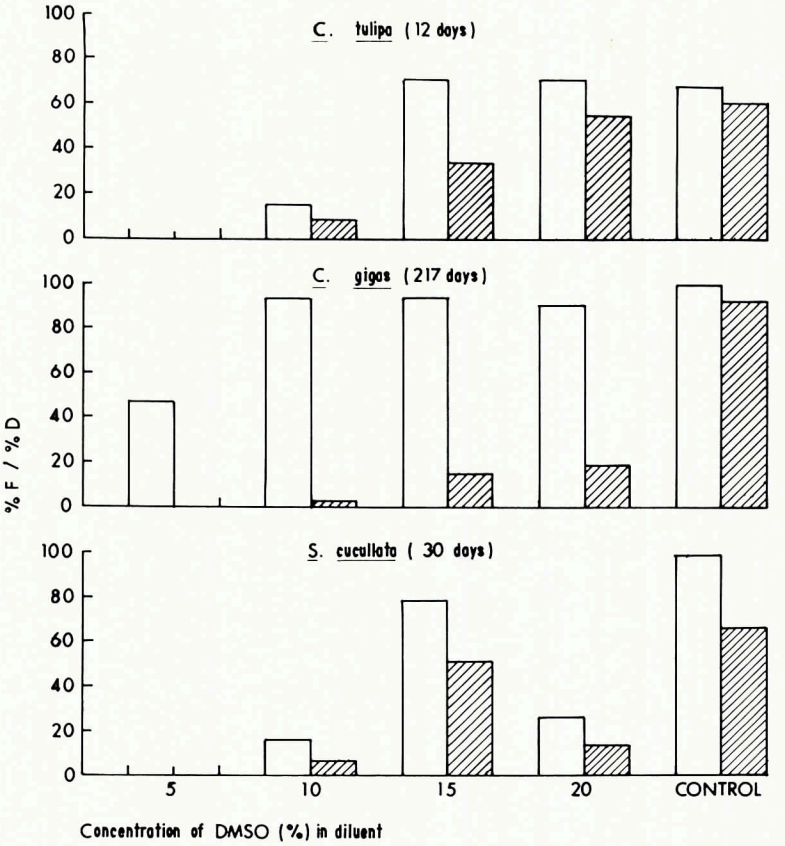

Fig. 1. Effect of the concentration of DMSO in the diluent on the viability of cold-stored (−190°C) spermatozoa of three species of oysters. Open bars, % fertilization; hatched bars, % larval yield. The lengths of storage period are indicated in parentheses.

were 20% for both *C. tulipa* and *C. gigas*, and 15% for *S. cucullata*, producing respective rates of larval yield of 54.7, 17.8 and 51.0%.

Motility values of the thawed spermatozoa of *C. iredalei* are shown in Table 1. It is seen that 10 and 15% DMSO provided satisfactory protection, resulting in 30.1 and 34.6% motility, respectively. Both of these values gave an equivalence of 3 on the 0–5 point scale of Legendre and Billard (1980).

Effect of glycine

Table 2 shows the effect of glycine as an additive to the diluent on the viability of the frozen spermatozoa of *C. tulipa*. A 2×3 factorial analysis of variance using log $(X+1)$ transformation and omitting 5% DMSO revealed that the influence of glycine was not significant on fertility of the spermatozoa ($P>0.1$). However, a significant enhancement ($P\simeq0.01$) was shown in the

TABLE 1

Motility of thawed spermatozoa of *C. iredalei* previously stored at −190°C for 168 days in diluent containing different concentrations of DMSO

DMSO concentration (%)	No. of spermatozoa counted	No. of motile spermatozoa	Motility (%M)	Equivalence of Legendre/ Billard Scale
5	2361	385	16.3	2
10	1537	462	30.1	3
15	1577	546	34.6	3
20	1384	148	10.7	1

TABLE 2

Effects of glycine (0.6%) as an additive to different concentrations of DMSO on the viability of spermatozoa of *C. tulipa* stored at −190°C for 7 days

DMSO	Without glycine (control)		With glycine		Compared to control (%)	
	%F	%D	%F	%D	Fertility	Larval yield
5	0.9	0.0	0.7	0.0	77.8	–
10	17.3	8.2	17.6	10.6	101.7	129.3
15	36.6	19.8	46.3	24.1	126.5	121.7
20	56.0	28.3	64.9	40.1	115.9	141.7

larval yield (Table 3) producing a barely significant interaction ($P=0.1$) with DMSO concentration.

Larval development

A summary of aspects of development of *C. tulipa* larvae obtained from fresh eggs fertilized with frozen spermatozoa is provided in Table 4. 'Control' cultures of larvae produced with fresh spermatozoa were included for comparison. It is evident that the experimental and control larvae were similar in the various aspects monitored. Growth rates of the experimental larvae ranged from 17.7 to 19.1 μm day^{-1} compared with 18.5 μm day^{-1} of the control. Larval shell indices were all approximately 1.2. Survival rates of the experimental larvae (87.3 to 92.6%) were comparable with the control (92.7%). Sixteen days after fertilization, 15.3 to 26.6% of the experimental larvae had attained competence while 25.4% of the control larvae were at the same stage of development.

TABLE 3

ANOVA table showing the effects of glycine additive and DMSO concentration on larval yield from fertilizations conducted with cold-stored (7 days) spermatozoa of *C. tulipa*

Source of variation	d.f.	SS	MS	F
Treatments	5	0.6059		
A. Additive	1	0.0353	0.0353	13.58**
B. Concentration	2	0.5682	0.2841	109.27***
A×B	2	0.0179	0.0090	3.46*
Error	6	0.0155	0.0026	
Total	11	0.6214		

*Significant at $P=0.1$; **significant at $P\simeq 0.01$; ***significant at $P<0.005$.

TABLE 4

Aspects of development of *C. tulipa* larvae 16 days after fertilization using spermatozoa frozen for 7 days at −190°C (The values are means for duplicate cultures except for 20% DMSO only)

Cryoprotectant used	Growth rate (μm day^{-1})	Shell height/ length index	Survival (%)	% With eyespots
15% DMSO only	17.7	1.20	91.8	15.3
20% DMSO only	18.0	1.24	92.6	19.5
15% DMSO+glycine	19.1	1.15	91.9	23.3
20% DMSO+glycine	18.9	1.20	87.3	26.6
Control (fresh sperm)	18.5	1.18	92.7	25.4

DISCUSSION

The spermatozoa of *C. tulipa*, *C. gigas*, *C. iredalei* and *S. cucullata* survived storage at −190°C, and those that were test-fertilized (see Fig. 1) demonstrated retention of fertility to varying degrees. Previous investigators of the cryopreservation of oyster spermatozoa who achieved high fertility rates (e.g. Zell et al., 1979: up to 91% and Bougrier and Rabenomanana, 1986: up to 80.7%) stored the SS in liquid nitrogen at −190°C. Staeger (1974), on the other hand, quoting unpublished data by Ott, that storage of oyster spermatozoa at −196°C proved detrimental, used −170°C as the storage temperature for *C. gigas* spermatozoa and achieved only up to 36.3% fertilization success. The results of the present work suggest that storage in liquid nitrogen at and around −190°C could be of wide application among oysters. The spermatozoa of all the four species of oysters used in this study were amenable to

the quick thaw technique; agreeing with the results of Robbins et al. (1976), Zell et al. (1979) and Legendre and Billard (1980).

The unavoidably wide differences in the storage periods of the frozen spermatozoa of the oysters preclude firm conclusions on the effects of DMSO concentration in the diluent (seawater – glycine, 0.6% – DMSO) on their viability. Nevertheless, the results (Fig. 1, Table 1) show different, but overlapping optimal concentrations in the range 10–20% for the four species. Whereas the viability of the spermatozoa of *C. gigas* covered this range, the optimal concentration for *C. tulipa* appeared to occur in the upper end of this range and those of *C. iredalei* (spermatozoa motility) and *S. cucullata* in the middle. This is an example of interspecific differences which probably derives from the fact that the action of cryoprotective agents on cells varies with the type and origin of the cells (Meryman, 1971). It is also apparent from the results that the optimal DMSO concentration for fertility may not necessarily be the same as for larval yield, as shown in the cases of *C. tulipa* and *C. gigas*. But it is of interest to note that in the latter species where the highest fertility (a mean of 93% in 10% DMSO) was achieved with the cryopreserved spermatozoa, the corresponding larval yields were the lowest among the three species tested. Bougrier and Rabenomanana (1986), using a diluent (DCSB4, pH 8.2)–cryoprotectant (DMSO, 10%), also achieved a high fertility of 80.7% with the spermatozoa of *C. gigas* but they did not provide information on larval yield. In the present study the wide disparity between fertilization rate and larval yield in *C. gigas* may suggest a possible ageing effect due to the prolonged period of storage (217 days) compared with 12 and 30 days for *C. tulipa* and *S. cucullata* respectively. Nevertheless, the 17.8% larval yield achieved with spermatozoa stored for over 7 months could be of immense practical significance for a species in which the female contains an average of 7.28 million eggs (see Van der Horst et al., 1985).

The results on the motility of the thawed spermatozoa of *C. iredalei* are quite important since Bougrier and Rabenomanana (1986) achieved fertilization rates varying from 13.0 to 80.7% with *C. gigas* spermatozoa of similar grades.

The apparent, but statistically insignificant, enhancement of the fertility of the spermatozoa of *C. tulipa* resulting from the addition of glycine to the cryoprotective diluent agrees with the finding of Staeger (1974). What needs to be explained is the significant improvement in the larval yield achieved with the glycine additive. According to Doebbler (1966), amino acids provide some degree of extracellular cryoprotection. Furthermore, oyster spermatozoa are more capable of utilizing endogenous reserve in the presence of glycine (see Staeger, 1974). Since DMSO is a cryophylactic agent which functions intracellularly (Doebbler, 1966; Rowe, 1966; Meryman, 1971) it is possible that the above attributes of glycine combined (synergistically) with that of DMSO

to improve the protection of the spermatozoa of *C. tulipa* which became even more significant when expressed in terms of larval yield.

Zell et al. (1979) reported vigorous growth of larvae from naturally spawned eggs fertilized with cryopreserved spermatozoa of *C. virginica*, but did not rule out latent freeze-induced injury in accounting for their low survival rate. In the present study, the information on growth rate, shell form, survival and attainment of competence of *C. tulipa* larvae obtained from extracted eggs fertilized with cryopreserved spermatozoa does not suggest the presence of latent freeze-induced injury during the 15 days of rearing. Furthermore, there appears to be no appreciable difference in the performance of the larvae with respect to the presence or absence of glycine in the cryoprotective diluent.

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REFERENCES

Bourgrier, S. and Rabenomanana, L.D., 1986. Cryopreservation of spermatozoa of the Japanese oyster, *Crassostra gigas*. Aquaculture, 58: 277–280.

Doebbler, G.F., 1966. Cryoprotective compounds. Review and discussion of structure and function. Cryobiology, 3: 2–11.

Ewart, J.W. and Epifanio, C.E., 1981. A tropical flagellate food for larval and juvenile oysters, *Crassostrea virginica* (Gmelin). Aquaculture, 22: 297–300.

Hughes, J.B., 1973. An examination of eggs challenged with cryopreserved spermatozoa of the American oyster, *Crassostrea virginica*. Cryobiology, 10: 342–344.

Lannan, J.E., 1971. Experimental self-fertilization of the Pacific oyster, *Crassostrea gigas*, utilizing cryopreserved sperm. Genetics, 68: 599–601.

Legendre, M. and Billard, R., 1980. Cryoconservation du sperme de truite arc-en-ciel (*Salmo gairdneri* R.). Bull. Fr. Pêche Piscicult., 278: 11–33.

Meryman, J.T., 1971. Cryoprotective agents. Cryobiology, 8: 173–183.

Robbins, R.K., Saacke, R.G. and Chandler, P.T., 1976. Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in French straws. J. Anim. Sci., 42: 145–154.

Rowe, A.W., 1966. Biochemical aspects of cryoprotective agents in freezing and thawing. Cryobiology, 3: 12–18.

Staeger, W.H., 1974. Cryobiological investigations of the gametes of the Pacific oyster *Crassostrea gigas*. M.S. Thesis, Oregon State University, Corvallis, OR, 45 pp.

Steel, G.D. and Torrie, J.H., 1960. Principles and Procedures of Statistics. McGraw-Hill, New York, NY, 481 pp.

Van der Horst, G., Dott, H.M., Samuels, J.E. and Genade, A., 1985. Short-term and long-term storage of viable oyster (*Crassostrea gigas*) sperm. S. Afr. J. Sci., 81: 404–405.

Walne, P.R., 1979. Culture of Bivalve Molluscs, 2nd edn. Whitefriars Press, London, 189 pp.

Yankson, K. and Moyse, J., 1983. Artificial fertilization and rearing of *Cerastoderma lamarcki* (Reeve) in the laboratory. J. Mollusc. Stud. Suppl., 12A: 209–213.

Zell, S.R., Bamford, M.H. and Hidu, H., 1979. Cryopreservation of spermatozoa of the American oyster (*Crassostrea virginica*). Cryobiology, 16: 448–460.